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PRINCIPAL INVESTIGATOR: Brian P. Dolan, OTHD

CONTRACTING ORGANIZATION: University of Maryland Baltimore County
Baltimore MD 21250

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Brian P. Dolan, OTHD

E-Mail: bdolan1@umbc.edu

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14. ABSTRACT: Genetic engineering of tumor cells to express MHC class II and subsequent use of said cells for treatment of established and metastatic tumors has yielded promising results in animal models for treatment of breast cancer. It is widely believed that the vaccine efficacy is due to the ability of such tumor cells to present tumor-specific antigens to CD4+ T helper cells which activate the immune system to eradicate tumors. Next generation cell-based vaccines will have enhanced antigen presentation capabilities to further stimulate the anti-tumor immune response. It has recently been proposed that MHC class II molecules physically localize to cell-surface microdomains, termed lipid rafts, to enhance antigen presentation. Further more, a correlation has been observed where cell-based tumor vaccines that have high levels of MHC class II in such rafts have higher efficacy than those with diminished or abolished levels of MHC class II in rafts. We propose to further target MHC class II molecules to lipid rafts to enhance the antigen presentation capabilities of tumor cell-based vaccines and than to use these modified vaccine cells for the treatment of established, metastatic disease in mouse models of breast cancer.

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Introduction

Genetically modified tumor cells designed to elicit host immune responses may have potential therapeutic benefit. These studies were undertaken to determine the mechanism by which tumor cells, genetically modified to express MHC class II molecules, enhance anti-tumor immune responses. The original hypothesis was that MHC II molecules physically localized to lipid rafts on the plasma membrane of tumor cells to facilitate presentation of antigens to $CD4^+$ T cells. However, as previously reported to this command, we were unsuccessful in further targeting MHC II molecules to lipid rafts in a murine mammary carcinoma cell line with the hopes of improving the efficacy of such cell-based vaccines. Concurrent studies being conducted with alternate tumor cell-based vaccines demonstrated that MHC class II molecules physically transferred from tumor cell-based vaccines to the hosts dendritic cells (DC), which used the in tact MHC II-peptide complex to activate $CD4^+$ T cells. We therefore extended these studies to 4T1 vaccine cells to determine if MHC molecules transferred to DC for T cell activation.

Body

Note: Text appearing in the modified Statement of Work (SOW) is underlined

Tasks 1-3 have been completed and previously reported to this command.

Task 4:Transfer of MHC class I and II molecules from 4T1, 4T1/A^d, and 4T1/CIITA cell lines to dendritic cells (DC), Months 24-36

a. Transfect the mammary carcinoma cell line 4T1 with genes encoding the alpha and beta chains of I-A^d or the class II transactivator gene (CIITA). **Completed**.
b. Determine if MHC class I and II molecules transfer from dead 4T1 cells to DC by lysing 4T1 cells from task 1a and culturing with DC from FVB (*H-2^q*) mice. DC will either be isolated as $CD11c^{\pm}$ cells from the spleen or derived by culturing bone marrow cells in GM-CSF and IL-4. **see below**
c. Measure MHC class I and II transfer by flow cytometry using fluorescent antibodies to both CD11c and either donor MHC class I (D^d) or donor MHC class II (I-A^d). **see below**

4T1 cells generated in task 4a were killed by repetitive freeze-thaw cycles until they were 100% dead as determined by trypan blue uptake. Lysed cells were then cultured with DC either isolated from the spleen of FVB (*H-2^q)* mice or with bone-marrow derived DC (hereafter BMDC)from FVB mice. DC were then purified to remove dead cells and analyzed by flow cytometry to determine if either MHC class I (D^d) or MHC class II (I-A^d) proteins were expressed on FVB DC (identified as cells expressing CD11c). As shown in figure 1A, $CD11c^+$ DC did express D^d protein after culture with BMDC DC, indicating that MHC class I molecules transferred from dead 4T1 cells to DC. No transfer was seen if DC were cultured with live 4T1 cells (data not shown). Similar

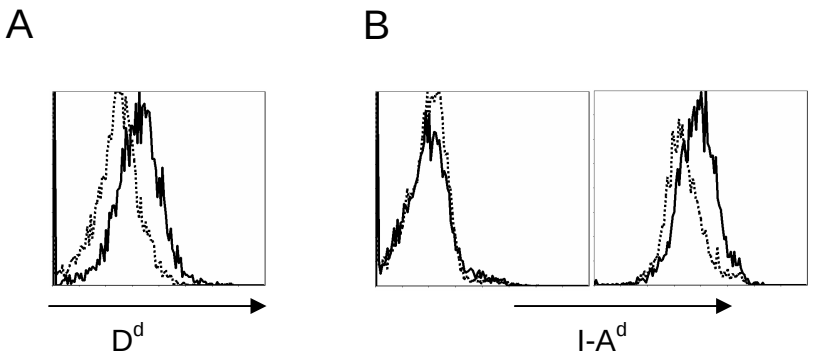

Figure 1. DC were cultured alone (dotted histogram) or with dead 4T1 cells (solid histogram), purified, and analyzed by flow cytometry. $CD11c^+$ cells were examined for etiher D^d or $I\text{-}A^d$ expression as indicated. *A.* BMDC were mixed with lysed 4T1 parental tumor cells. *B.* Splenic DC were cultured with either $4T1/A^d$ expressing tumor cell-based vaccines or 4T1/CIITA cells. In all histograms relative cell number is displayed on the y-axis

results were obtained if splenic DC were used (data not shown).

To test whether MHC class II similarly transferred to DC, we mixed splenic DC with either 4T1 cells genetically modified to express $I\text{-}A^d$ ($4T1/A^d$) or 4T1 cells expressing $I\text{-}A^d$ due to genetic modification of expression of the class II transactivation gene (CIITA). As shown in figure 1B, $I\text{-}A^d$ was only detected on recipient DC following culture with lysed 4T1/CIITA cells, and not $4T1/A^d$ cells. CIITA expression not only drives expression of MHC class II molecules but also invariant chain (Ii), which is detrimental to the vaccine efficacy afforded by MHC class II expression by tumor cells. Because MHC II transfer to DC was not observed when vaccine cells were used as MHC II donors, it is possible that the diminished vaccine efficacy of such cells is due to the lack of MHC class II transfer.

Task 5: Determine the effect of IFN-γ pre-treatment of 4T1 cells on MHC class I and class II transfer to DC, Months 24-36.

a. Treat 4T1 cells with 100 units/ml recombinant IFN-γ for two days to upregulate both MHC class I and class II expression on 4T1 cells.
b. Measure MHC class I and II expression on IFN-γ treated cells.
c. Compare transfer of MHC class I and class II proteins from IFN-γ treated and non-treated controls to DC as described in task 1.

Treatment of parental cells with recombinant murine IFN-γ resulted in up-regulation of MHC class I, but not MHC class II molecules (data not shown). Therefore, transfer of MHC class I from IFN-γ treated 4T1 cells was compared to transfer from non-treated cells. As shown in figure 2, D^d molecules were detected on FVB-derived BMDC cultured with either IFN-γ treated or non-treated cells, however levels of D^d were approximately two times greater when donor 4T1 cells were treated with IFNγ. These data indicate that enhanced MHC class I expression on donor cells leads to greater transfer of MHC molecules to DC. If correct, this observation could lead to better cell-

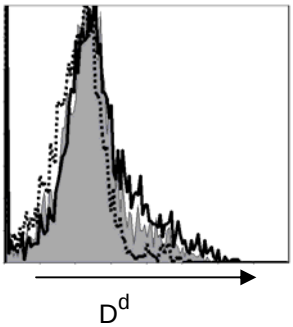

Figure 2. 4T1 cells were culture without or with 100 units IFN-g for two days. Cells were subsequently lysed and mixed with FVB-derived BMDC. BMDC DC were then analyzed for D^d expression by flow cytometry. BMDC cultured alone (dotted histogram), with untreated 4T1 (shaded histogram), or with IFN-γ treated (solid histogram) are shown.

based vaccine development whereby patients tumor cells are treated with IFN-γ to enhance expression of cognate MHC class I-peptide complexes, which would be subsequently transferred to patient derived DC. DC could be reintroduced into the patient, and potentially, activate greater numbers and/or more diverse anti tumor $CD8^+$ T cells.

Task 6: Determine if intact MHC class I-peptide complexes transfer from 4T1 cells to DC for $CD8^{\pm}$ T cell activation, Months 24-36

a. Transfect 4T1 cells with genes for ovalbumin (ova) and the allogenic MHC class I K^b to generate 4T1/ova/K^b cells. Additionally, transfect 4T1 cells with genes for influenza Hemaglutinin (HA) to generate 4T1/HA cells.
b. Transfer either K^b-ova complexes from 4T1/ova/K^b cells or K^d-HA complexes from 4T1/HA cells to FVB bone-marrow derived DC as in task 1. Purify DC from dead 4T1 cells by passage over ficol.
c. Culture purified DC with either transgenic OT-1 $CD8^{\pm}$ T cells (restricted to K^b-ova) or transgenic clone 4 $CD8^{\pm}$ T cells (K^d-HA restricted). Measure CD69 upregulation on T cells as well as release of IL-2 and IFN-γ by ELISA.

Attempts to transfect and screen 4T1 cells with the model antigens ova and HA were unsuccessful. Because of this, task 6 can not be completed as described (see additional work below).

Additional Work

Because we were unsuccessful in generating 4T1 cells expressing model antigens, we used alternative tumor systems to study the transfer of MHC class I-peptide complexes from tumor cells to DC and the subsequent activation of $CD8^+$ T cells by recipient DC. The results of these experiments are detailed in appendix B and were published in the Journal of Immunology (see reportable outcomes). Briefly, the T cell lymphoma EL4 and the immortalized fibroblast cell line DAP (both expressing ova) were used to measure transfer of MHC class I-peptide complexes to DC and the subsequent activation of T cells. We found that peptide-MHC complexes generated by donor cells could be acquired by recipient DC and, without further processing, be used to activate transgenic $CD8^+$ T cells. Additionally, we demonstrate both in vivo and in vitro that DC which have acquired intact peptide-MHC complexes are better activators of transgenic T cells than

DC simply cross-presenting antigen. These results suggest a novel method by which DC activate $CD8^+$ T cells and offers a new avenue for vaccine development. The process of peptide-MHC complex transfer has been termed "DC cross-dressing."

Key Research Accomplishments:

- Transfer of MHC class I molecules from mammary carcinoma cell lines to DC has been demonstrated, a process enhanced by pretreatment of tumor cells with IFN-γ.

- Using alternate tumor systems, we have demonstrated that intact peptide-MHC complexes transferred to DC can be used in the activation of $CD8^+$ T cells, and this process leads to enhance activation of T cells compared to cross-presentation of antigens alone.

Reportable Outcomes:

- A manuscript entitled "Tumor-specific CD4+ T cells are activated by "cross-dressed" dendritic cells presenting peptide-MHC class II complexes acquired from cell-based cancer vaccines." was published in the Febuary 1, 2006 issue of the *Journal of Immunology* and is attached to this report as appendix A.
- A manuscript entitled "Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells" was published in the November 1, 2006 issue of the *Journal of Immunology* and is attached to this report as appendix B.
- A short talk entitled "Dendritic Cells "Cross-Dressed" with Peptide-MHC Class I Complexes from Necrotic Cells Activate CD8+ T Cells" was given at the Keystone Symposia on Viral Immunity in March 2006.
- A short talk entitled "Dendritic Cells "Cross-Dressed" with Peptide-MHC Class I Complexes from Dead Cells Activate CD8+ T Cells" was given at the AAI meeting in Boston in May 2006.

Conclusions

The goal of this project was to understand how genetically modified tumor cells expressing MHC class II proteins activate the immune system to facilitate tumor rejection. Initially, we believed that physical localization of MHC II proteins to cell-surface lipid rafts was responsible for activation of $CD4^+$ T cells. Studies to corroborate this hypothesis fell short of expected goals and led the project in a different direction. The different direction, however, revealed a novel mechanism by which T cells can be activated. This finding helps to explain how MHC class II^+ tumor cell-based vaccines illicit and immune response and will eventually lead to the development of better cell-based vaccines for the prevention and treatment of malignancies.

Appendix A

Tumor-specific $CD4^+$ T Cells are Activated by "Cross-Dressed" Dendritic Cells Presenting Peptide-MHC Class II Complexes Acquired from Cell-based Cancer Vaccines[1]

By

Brian P. Dolan, Kenneth D. Gibbs Jr., and Suzanne Ostrand-Rosenberg[2]

Department of Biological Sciences

University of Maryland Baltimore County, Baltimore MD 21228

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Footnotes:

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[2] Address correspondence and reprint requests to Dr. S. Ostrand-Rosenberg, Department of Biological Sciences, U. of Maryland, 1000 Hilltop Circle, Baltimore, MD 21250.

[3] Abbreviations used in this study:

BMDC, bone marrow-derived dendritic cell

DC, dendritic cell

DiD, 1,1" dioctadecyl 3,3,3', 3' tetramethylindodicarbocyanine

DTR, diphtheria toxin receptor

DTx, diphtheria toxin

HEL, hen egg lysozyme

pMHC, peptide-MHC

Abstract

Tumor cells that constitutively express MHC class I molecules and are genetically modified to express MHC class II and costimulatory molecules are immunogenic and have therapeutic efficacy against established primary and metastatic cancers in syngeneic mice, and activate tumor-specific human $CD4^+$ T lymphocytes. Previous studies have indicated that these MHC II vaccines enhance immunity by directly activating tumor-specific $CD4^+$ T cells during the immunization process. Because dendritic cells (DC) are considered the most efficient antigen presenting cells, we have now examined the role of DC in $CD4^+$ T cell activation by the MHC II vaccines. Surprisingly, we find that DC are essential for MHC II vaccine immunogenicity; however, they mediate their effect through "cross-dressing." Cross-dressing (or peptide-MHC transfer) involves the generation of peptide-MHC (pMHC) complexes within the vaccine cells, and their subsequent transfer to DC, which then present the intact, unprocessed complexes to $CD4^+$ T lymphocytes. The net result is that DC are the functional APC; however, the immunogenic pMHC complexes are generated by the tumor cells. Since MHC II vaccine cells do not express the MHC II accessory molecules invariant chain and DM, they are likely to load additional tumor antigen epitopes onto MHC II molecules, and therefore activate a different repertoire of T cells than DC. These data further the concept that transfer of cellular material to DC is important in antigen presentation, and they have direct implications for the design of cancer vaccines.

Introduction

The potential efficacy of immunotherapy for the treatment of cancer has led to the development of novel experimental strategies. Many of these innovative approaches focus on the activation of tumor-specific T lymphocytes because the cell-mediated arm of the immune system can be effective in destroying tumor cells and in providing long-term protection (memory) against the recurrence and/or outgrowth of primary and/or metastatic tumor cells (1-3). Since optimal activation of tumor-specific, cytotoxic $CD8^+$ T cells requires co-activation of $CD4^+$ helper T lymphocytes, we are developing cell-based cancer vaccines that activate both $CD4^+$ and $CD8^+$ T cells. Many tumor cells constitutively express MHC class I molecules and are therefore capable of interactions with $CD8^+$ T cells. Several studies have shown that co-expression of T cell costimulatory molecules in the tumor cell is sufficient for activation of tumor-specific $CD8^+$ T cells (4-8). However, these cells do not co-express MHC class II molecules, and therefore cannot activate $CD4^+$ T cells to provide the requisite help. We have reasoned that genetic manipulation of MHC class I^+ tumor cells to co-express MHC class II and T cell costimulatory molecules would create a cell-based vaccine ("MHC II vaccine") that is capable of activating tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes (9-11).

In vivo studies with three independent mouse tumors (Sa1 sarcoma, B16 melanoma, and 4T1 mammary carcinoma) have shown that vaccination/treatment with MHC II vaccines provides prophylactic protection (11), mediates rejection of established primary tumor (9), and reduces established spontaneous metastatic disease while extending survival (12). Although the MHC II vaccines have not as yet been tested clinically, in vitro studies demonstrated that HLA-DR and CD80-modified human ocular

melanoma and breast cancer cells are potent activators of tumor-specific HLA-DR and HLA-A-matched $CD4^+$ and $CD8^+$ human T cells, suggesting that the vaccines may also be effective in patients (13, 14).

The MHC II vaccines were designed to facilitate T cell activation by direct presentation of endogenously synthesized tumor antigens and by-pass the need for host APC to capture and present tumor antigens. Several lines of evidence support the premise that the vaccine cells are the relevant APC in vivo: 1) Bone marrow chimeric mice and nude mice inoculated with genetically disparate vaccine cells develop tumor-specific $CD4^+$ T cells restricted to the genotype of the tumor (15, 16); 2) Vaccine cells activate naïve T cells in vitro (17); and 3) Co-expression of the MHC class II chaperone protein invariant chain (Ii), which blocks loading of endogenous antigens onto MHC class II molecules, blocks both in vitro presentation of endogenous antigens and abolishes vaccine efficacy (16, 18, 19). Although there is also evidence from other systems that tumor cells directly activate T cells (8, 20), most studies indicate that dendritic cells (DC) are the principle APC that activate tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes (21, 22). DC activate T cells by capturing exogenous antigens released from tumor cells, processing them into peptides, and presenting the peptides to T cells via the process of cross-presentation. Because of their superior antigen presentation capabilities, DC are considered the most important APC in vivo.

Although our previous experiments indicated that MHC II vaccine cells are the principle APC during vaccination (15, 16), these studies do not exclude a role for DC in MHC II vaccine efficacy. We now report that DC are essential for MHC II vaccine efficacy; however, they mediate their effect through a novel mechanism that is distinct

from cross-presentation. The process involves the generation of peptide-MHC (pMHC) complexes within the vaccine cells, and their subsequent transfer to DC, which then present the intact, unprocessed complexes to $CD4^+$ T lymphocytes. This process of "cross-dressing" produces DC which are the functional APC; however, the pMHC complexes that are presented are generated by the MHC II vaccine cells. This result is consistent with our earlier findings that MHC II vaccine genotype governs $CD4^+$ T cell restriction, and provides a mechanistic explanation for the potent therapeutic efficacy of the MHC II vaccines.

Materials and Methods

Mice

Original breeding stocks of A/J, BALB/c, and Itgax-DTR/EGFP (CD11c-DTR) (23) transgenic mice were from The Jackson Laboratory (Bar Harbor, ME). Original breeding stock of female FVB mice (3-6 months of age) were from Charles River Labs (Cambridge, MA). 3A9 transgenic mice (24) were maintained on a C3H/HeJ background or crossed once with A/J mice to yield (C3H-3A9 x A/J)F1 mice. Offspring were screened as described (17). F1 generations of CD11c-DTR mice were obtained by mating female transgenic mice on a BALB/c background with male A/J mice. Offspring were screened for transgene expression as described (23). Mice were housed according to the NIH guidelines for the humane treatment of laboratory animals and bred in the University of Maryland Baltimore County (UMBC) animal facility. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Cells and Antibodies

SaI (11), SaI/HEL (19), SaI/A^k(11), SaI/A^k/HEL (19), MELF10/A^b (25), 4T1 (12), and B16.BL6/A^b (26) tumor cells were grown as described. The I-A^k-restricted, HEL-specific T cell hybridomas A2.A2 (27) and 3B11.1 (28), and the B cell lymphoma M12.C3.F6 (29) were grown as described (16, 30). The B cell lymphoma A20 was grown in RPMI (Biofluids, Rockville MD) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan UT), 1% penicillin, 1% streptomycin, 1% gentamicin (Biofluids), and 1% Glutamax (Life Technologies, Rockville MD). 3LL tumor cells were

grown in DMEM (Biofluids) supplemented with 10% fetal clone I serum (Hyclone), 1% penicillin, 1% streptomycin, 1% gentamicin, and 1% glutamax. The following antibodies directly coupled to FITC or PE were purchased from BD Pharmingen (San Diego, CA): CD11c (clone HL3), I-A^k (11-5.2), I-A^d (39-10-8), I-A^b (AF6-120.1), I-A/I-E (2G9), K^k (36-7-5), K^b (AF6-88.5), D^d (34-2-12S), CD80 (1G10), and CD86 (GL1). CD4-PE was purchased from Caltag (Burlingame, CA). All purchased antibodies were diluted 1:50 in 0.5% BSA in PBS for staining. B220 and HO13-4-9 were used as undiluted culture supernatants of hybridoma cells. 10-2.16 and 3JP antibodies were purified from hybridoma culture supernatants and used as described (19). For some experiments, 10-2.16 antibody was directly coupled to Alexa 488 (Molecular Probes, Eugene OR) following the manufacturer's protocols; 3 moles of dye incorporated per mole of antibody.

Tumor Challenges

(CD11c-DTR x A/J)F1 transgenic and non-transgenic littermates were injected i.p. with diphtheria toxin (DTx) at 5 ng/g body weight (Sigma, St.Louis MO) or left untreated, and eight hours later inoculated i.p. with 8 x 10^5 SaI or SaI/A^k tumor cells. In some experiments, approximately 5 x 10^6 $CD8^+$ T cells from non-transgenic littermates were injected i.v. into the tail vein of transgenic mice 24 hours before DTx administration and tumor challenge. The SaI variant used in these studies is an ascites tumor that grows progressively in A/J mice and is lethal before day 20 after tumor inoculation. Tumor inoculated mice were monitored every other day by visual inspection for development of ascites tumor and were sacrificed when they became moribund. Tumor incidence is the

number of mice that developed lethal ascites tumors and were moribund by day 20, divided by the total number of mice inoculated. In some instances, male CD11c-DTR mice developed a solid tumor which caused paralysis of one hind leg. Such tumors caused mice to become moribund an average of 37 days after tumor inoculation. Since these tumors resulted in animals becoming moribund long after the ascites tumors were lethal and tumor growth was drastically altered from the parental tumor growth pattern, these mice were not considered susceptible to ascites tumors.

Dendritic cell isolation

DC were purified from the spleens of 3-6 month old mice by magnetic bead sorting. Spleens were harvested, minced, and incubated in collagenase D (Sigma) for 30 minutes at 37°C. Resulting cells were washed in serum-free RPMI medium and red blood cells were depleted by treatment with ACK lysing buffer (Biofluids). Splenocytes were resuspended in 0.5% BSA in PBS (1-2 x 10^8 cells/ml), incubated for 30 minutes on ice with 100 μl CD11c microbeads (Miltenyi Biotech, Auburn CA) per spleen, and applied to a magnetic column to isolate $CD11c^+$ cells. Purified $CD11c^+$ cells were cultured for 1 hour at 37° C in DC medium (IMDM supplemented with 5% FCS, 1% penicillin, 1% streptomycin, 1% gentamicin, and 1% Glutamax), followed by a gentle washing with warm DC medium to remove residual dead cells and contaminating lymphocytes. Following purification, cells were routinely 80-90% $CD11c^+$. Bone-marrow derived DC (BMDC) were obtained by isolating marrow from the femurs of 6 month old mice. Red blood cells were removed by treatment with ACK lysing buffer. B cells, T cells, and MHC class II^+ cells were depleted by incubation with approximately

1μg each of B220, HO-13-4-9, and 3JP antibodies, followed by addition of rabbit complement (Cedarlane, Ontario Canada) as described (31). Remaining cells were re-suspended at 10^7 cells/ml in BMDC medium (RPMI supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1% gentamicin, 1% Glutamax, 5 x 10^{-4} M 2-mercaptoethanol (Sigma), 20 ng/ml recombinant GM-CSF and 10ng/ml recombinant IL-4 (both from RDI, Flanders NJ)) and cultured at 37° C in 5% CO_2. Media was replaced every two days and loosely attached cells removed. After six to eight days of culture, cells were routinely greater than 70% $CD11c^+$ as measured by flow cytometry.

MHC transfer experiments

Cells were freeze-thaw lysed by freezing 10^7 cells/ml of serum-free RPMI media at -80° C for 20 minutes, followed by rapid thawing at 37° C. The cycle was repeated up to 3 times until lysis was complete as determined by trypan blue uptake. Osmotic lysates of cells were obtained by re-suspending 5 x 10^6 cells/ml in 0.9 volume of distilled water for 30 minutes on ice, followed by the addition of 0.1 volume of 10x PBS. Cells were heat killed by re-suspending to 5 x 10^6 cells/ml in PBS and incubation at 65° C for 30 minutes. Freeze-thawed, osmotically lysed, or heated killed tumor cells were added to DC that had been plated one hour earlier in 6 well dishes at 1-2 x 10^6 cells/well/2 ml DC media at a ratio of 2-4 tumor cells per DC. Following a three hour incubation at 37^o C in 5% CO_2, tumor cell material was removed from the cultures by extensive washing of the attached DC with PBS. For confocal experiments, tumor cells were labeled before freeze thaw lysis with the lipophilic dye 1,1" dioctadecyl 3,3,3', 3' tetramethylindodicarbocyanine (DiD Molecular Probes, excitation 648 nm, emission

670 nm) according to the manufacturer's recommendations. For experiments with BMDC tumor cell material was removed after the three hour incubation by centrifugation at 1100 g through Ficoll-Paque (Pharmicia-Biotech, Uppsala Sweden) for 15 minutes at room temperature. DC were recovered at the Ficoll-Paque-media interface. For transwell experiments, 1-2 x 10^6 DC were plated in 2 ml DC media in the bottom chamber of an 8 μm transwell (Corning, Corning, NY) and 2-3 cell equivalents of freeze-thawed tumor cells in 1 ml of serum-free RPMI were added to the top chamber. In some experiments, 1.0 μm FITC-beads (Polyscience, Inc., Warrington, PA) were also added to the top chamber.

CFSE labeling and pronase treatments

BMDC were treated with 3 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes) at 1 x 10^7 DC/ml in PBS for 10 minutes at room temperature. The reaction was quenched by the addition of calf serum and washing with excess PBS. DC were treated with pronase (Type XIV, Sigma, St. Louis MO) by incubating 10^6- 5 x 10^6 DC with 2mg/ml pronase in PBS for 20 minutes at 37°C. Treated cells were washed extensively with DC media.

Flow cytometry and confocal microscopy

Cells were labeled for immunofluorescence and analyzed by flow cytometry using a Coulter XL instrument as described (32) and analyzed using Coulter Expo32 ADC software. For confocal microscopy, DC were fluorescently labeled with CD11c-PE and I-A^k-Alexa 488 antibodies, washed with PBS to remove unbound antibody, and then

adhered to poly-L-lysine (Sigma) coated slides for 30 minutes at 4°C. Attached cells were fixed by a 5 min incubation with 2% paraformaldehyde in PBS. Fixative was quenched by the addition of 1M glycine (Sigma) and slides were washed once with excess PBS and once with excess water. Coverslips were mounted with 20 μl of a 50% solution of Fluoromount-G (Southern Biotechnologies, Birmingham AL) in PBS. Slides were visualized using a Zeiss LSM 510 Meta Confocal Microscope located at the Johns Hopkins University Integrated Imaging Facility and analyzed using LSM image browser software. Fluorescent images were collected sequentially with each scan repeated four times and then averaged. Approximately 1 μm optical slices through the z-plane were collected. A minimum of 25 labeled cells was analyzed for each cell type for each data set.

T cell isolation and antigen presentation

$CD4^+$ 3A9 TCR transgenic T cells were isolated by negative selection using the $CD4^+$ T cell isolation kit according to the manufacturer's directions (Miltenyi Biotech). Purified populations were greater than 90% $CD4^+$ as measured by flow cytometry. Antigen presentation assays using DC as APC were performed as previously described for tumor cells (33) with the following modifications. Splenic DC (1×10^5 cells/well) were incubated with 2.5 - 3 times as many freeze-thawed tumor cells. Following a three hour incubation (37^o C, 5% CO_2), wells were washed extensively with PBS to remove tumor cell material, and 200 μl of RPMI media supplemented with 10% fetal calf serum was added to each well. Antigen presentation experiments with hybridomas (5 x

10^4/well) (33) and with transgenic T cells (10^5/well) (17) were performed as described. IL-2 release was quantified by ELISA (Endogen, Rockford IL) as described (33).

In vivo T cell priming and in vitro measurement of T cell activation

(CD11c-DTR x A/J)F1 mice were untreated or injected i.p. with 5ng /g body weight DTx and 6 hours later immunized i.p. with either 8 x 10^5 SaI/HEL or SaI/A^k/HEL cells. Six days later splenic $CD4^+$ T cells were isolated by negative selection using magnetic beads according to the manufacturer's directions (Miltenyi Biotech). Purified cells were > 90% $CD4^+$ as measured by flow cytometry. In vivo priming of purified $CD4^+$ T cells was assessed by measuring their in vitro response to HEL presented by professional APC as described (16), with the following modifications. One million purified $CD4^+$ T cells were cultured overnight with 10^5 M12.C3.F6 cells which had previously been pulsed for 15 hours with 2mg/ml HEL protein (Sigma, St.Louis MO) in RPMI medium containing an additional 1 mg/ml HEL. IL-2 release was determined by ELISA (Endogen, Rockford IL). Background levels of IL-2 were determined by culturing T cells without APC and were subtracted from experimental values.

Statistical analyses

Standard deviations and Student's T test were calculated using Microsoft Excel 2002.

Results

DC are required for efficacy of MHC II vaccines

To determine if host DC are required for MHC II vaccine-mediated T cell activation and tumor rejection, mice were depleted for DC prior to tumor inoculation and observed for tumor progression. CD11c-DTR transgenic mice express the simian diphtheria toxin receptor (DTR) under control of the DC specific CD11c promoter. When CD11c-DTR mice are given diphtheria toxin (DTx), their DC are deleted for two days and return to normal levels by day 5 (23). The mouse SaI sarcoma and its corresponding MHC II vaccine, SaI/A^k, are derived from A/J mice. Therefore, CD11c-DTR mice (BALB/c background) were mated to A/J mice to produce offspring semi-syngeneic to SaI. When challenged i.p. with live wild type SaI cells, CD11c-DTR and non-transgenic littermates developed ascites tumor and were moribund by day 20 (Table 1). In contrast, neither transgenic nor non-transgenic littermates developed ascites tumor after inoculation of live SaI/A^k vaccine cells (Table 1), in agreement with previous studies showing that expression of syngeneic MHC II enhances tumor cell immunogenicity. However, if DC are deleted from the CD11c-DTR mice by a single dose of DTx six hours prior to tumor challenge, then SaI/A^k cells cause a lethal ascites tumor, demonstrating that DC are necessary for rejection of MHC II vaccine cells.

Since CD11c expression has been detected on activated $CD8^+$ T cells (23), it is possible that DTx-treated CD11c-DTR mice develop tumors because they are CD8-depleted. To control for this possibility $CD8^+$ T cells from non-transgenic littermates were adoptively transferred to CD11c-DTR mice prior to DTx administration and tumor

challenge. These reconstituted mice were also susceptible to SaI/A^k tumor growth (data not shown), indicating that tumor susceptibility was due to DC depletion.

DC acquire MHC class II molecules from dead tumor cells.

The dual requirement for MHC class II expression by tumor cells and the presence of host DC suggested that interactions between these two cell populations might be essential for maximum T cell activation. Since recent reports have shown the physical transfer of plasma membrane molecules between multiple cell types, including DC (34-36), we examined if MHC class II proteins could transfer from tumor cells to DC. Splenic DC were obtained from FVB mice (*H-2*^{q}) by magnetic bead selection, incubated with SaI or SaI/A^k cells for 3 hours, and then analyzed for I-A^k expression using an I-A^k-specific mAb, (11-5.2) and flow cytometry. When FVB DC were incubated with live SaI or SaI/A^k tumor cells, no I-A^k expression is seen on the DC (figure 1A). However, if FVB DC are mixed with lysates of freeze-thawed SaI/A^k tumor cells then the DC acquire I-A^k at their cell surface. Incubation with freeze-thawed SaI cells does not result in DC expression of I-A^k. Transfer of I-A^k molecules was confirmed using a second I-A^k-specific mAb (10-2.16) and was also seen when recipient BALB/c splenic DC were used (data not shown). Transfer of I-A^k molecules from SaI/A^k vaccine cells to FVB DC was also seen when tumors were killed by osmotic lysis, but not when cells were heat killed (data not shown), suggesting that cells must be disrupted for transfer to occur.

To eliminate artifacts due to contamination of the DC population with freeze-thawed or residual live SaI/A^k cells, DC were adhered to plastic and washed extensively to remove tumor cell debris prior to labeling for flow cytometry. In addition, DC and

SaI/A^k cells have different forward scatter and side scatter profiles allowing for precise bitmapping of the DC and elimination of SaI/A^k cells and/or material (figure 1B). DC were also distinguishable from SaI/A^k material because they are PI negative, while freeze-thawed SaI/A^k cells are PI positive (figure 1B).

Transfer of MHC class II to DC may represent a transient display of MHC class II molecules that occurs during endocytosis. To determine if the observed transfer events were transient, FVB DC were exposed to freeze-thawed SaI/A^k cells for three hours and then extensively washed to remove tumor debris as in figures 1A and 1B. DC were then incubated in media containing GM-CSF and IL-4 and analyzed for I-A^k expression 20 hours and 48 hours later. As seen in figure 1C, DC expressed tumor-encoded MHC class II molecules during the 48 hour incubation indicating that MHC II transfer is probably not due to transient endocytosis. DC also underwent maturation during the 48 hour incubation as determined by the up-regulation of the costimulatory molecules CD80 and CD86 (figure 1D), demonstrating that transferred MHC class II was retained and stably expressed during the maturation process.

To ascertain if MHC II transfer is restricted to splenic DC, experiments were performed with bone marrow-derived DC (BMDC). FVB BMDC were obtained by incubation of bone marrow cells in the presence of GM-CSF and IL-4 for 6-8 days until cells were greater than 70% $CD11c^+$ as assessed by flow cytometry. The resulting cells were then incubated with lysates of freeze-thawed SaI/A^k cells as in previous experiments with splenic DC, and transfer of I-A^k molecules to BMDC was assessed by flow cytometry. As seen in figure 1E, tumor-derived I-A^k molecules are also transferred to BMDC.

MHC class II transfer occurs from a variety of tumor cells

To determine if MHC class II transfer from tumor cells to DC is a general phenomenon, we examined if other MHC II vaccine cells and class II positive tumor cells could be donors. The B16 melanoma derived cell lines B16.BL6 and MELF10 were previously transfected with I-A^b genes and shown to have decreased tumorigenicity and to activate immune responses (25, 26). When either cell type is lysed by freeze-thawing and mixed with FVB splenic DC, I-A^b molecules are detected on the surface of recipient DC as determined by flow cytometry (figure 2A, upper panels). In agreement with the data presented for SaI/A^k no I-A^b is detected when live tumor cells are used (data not shown). Similarly, I-A^d and I-A^k molecules are transferred from the B cell lymphoma lines A20 and M12.C3.F6, respectively, following incubations of these freeze-thawed cells with FVB DC (figure 2A, lower panels). Therefore, MHC class II transfer from dead tumor cells to DC appears to be characteristic of multiple tumors and is not restricted by tissue type or MHC class II genotype.

MHC class I transfers from necrotic tumor cells to DC

To determine if MHC class I molecules are similarly transferred and retained, three independent tumor cell lines with different MHC class I genotypes were tested. FVB splenic DC were incubated with lysates of freeze-thawed 4T1 mammary carcinoma (D^d), 3LL Lewis lung carcinoma (K^b), or SaI sarcoma (K^k) and analyzed for transferred MHC class I molecules using the same procedure as used for the MHC II experiments. As shown in figure 2B, tumor MHC class I expression is detected on recipient DC.

Transferred MHC class I was stably expressed for up to two days (data not shown). These data indicate that like MHC class II, MHC class I molecules can be transferred from necrotic tumor cells to DC.

Transfer of MHC class II molecules is cell contact dependent and requires DC surface receptors.

To determine if direct contact between donor tumor cells and recipient DC was necessary for MHC class II transfer, or if MHC II transfer was mediated through soluble factors or exosomes, FVB DC were placed in the bottom half of a transwell chamber containing an 8 μm semi-permeable membrane, and freeze-thawed lysates of SaI/A^k tumor cells were placed in the top half. Following a three hour incubation, DC were analyzed for I-A^k expression by flow cytometry. To ensure soluble material could traverse the semi-permeable membrane, one μm FITC-coated latex beads were added to the tumor lysate in the upper chamber. In the presence of the semi-permeable membrane, MHC class II transfer to DCs is blocked (figure 3A) while DC uptake of FITC-coated latex beads is only 16% less than uptake in the absence of the membrane. While exosome-mediated transfer of MHC class II can not be ruled out, these data suggest that transfer of MHC class II molecules from tumor cells to DC requires cell-to-cell contact or exposure to > 8 μm tumor-derived particulate matter.

Acquisition of molecules by DC can occur by several mechanisms, including receptor-mediated endocytosis or macropinocytosis. If surface receptors are necessary for MHC class II transfer to DC, then treatment of DC with pronase, a protease which destroys surface receptors without affecting macropinocytosis (37), should eliminate

MHC class II transfer. CFSE-labeled BMDC were pronase treated prior to incubation with freeze-thawed lysates of SaI/A^k cells and subsequent analysis for I-A^k transfer by flow cytometry. BMDC were used in these experiments because they were more resistant to CFSE labeling and pronase treatment then splenic DC. As is shown in figure 3B, pronase treatment inhibited transfer of MHC class II from tumor cells to DC. As a control to demonstrate that pronase treatment does not significantly affect non-receptor-mediated uptake, pronase-treated BMDC were incubated with FITC-dextran, which is internalized predominantly by non-receptor–mediated processes. Pronase treatment diminished dextran uptake by 17% as measured by flow cytometry (data not shown), indicating that non-receptor-mediated uptake was largely unaffected. These results indicate that MHC II transfer to DC requires expression of DC cell-surface proteins and is not mediated by bulk fluid uptake by the DC.

DC acquire discrete regions of tumor plasma membrane containing MHC class II

DC can acquire plasma membrane lipids from other cells (34). To determine if plasma membrane lipids from SaI/A^k vaccine cells transferred to DC, confocal microscopy was used. Splenic DC from FVB mice were exposed to freeze-thawed lysates of SaI or SaI/A^k cells whose plasma membranes were previously labeled with the fluorescent lipophilic dye DiD. After transfer, the DC were stained with mAbs to I-A^k and CD11c. DC incubated with labeled SaI/A^k lysates contain discrete patches of co-localized tumor-derived lipid and MHC II molecules or patches of lipid without MHC II, while DC incubated with SaI lysates exclusively contain transferred lipid devoid of I-A^k (figure 3C). To determine the stability of transferred MHC class II over time, FVB DC

exposed to SaI/A^k lysates for 3 hours were extensively washed to remove tumor material and either stained for CD11c and I-A^k or were incubated in GM-CSF and IL-4 for 48 hours and then stained and visualized. As seen in figure 3D, patches of transferred I-A^k are detected on the recipient DC immediately after transfer and by 48 hours I-A^k is dispersed over the DC cell surface.

Preformed MHC class II-peptide complexes transfer to DC and activate $CD4^+$ T cells

If MHC II transfer from genetically modified tumor cells to DC is necessary for immunogenicity, then recipient DC should activate T cells to tumor encoded antigens restricted to the MHC genotype of the tumor cells. To test this hypothesis, we asked if DC containing transferred MHC class II-tumor antigen peptide complexes activate tumor specific $CD4^+$ T cells restricted to the tumor cell genotype and specific for tumor-encoded antigen. SaI/HEL or SaI/A^k/HEL cells were killed by freeze-thaw lysis and cultured with FVB splenic DC as in previous experiments. DC were then washed extensively to remove residual tumor material and cultured with I-A^k-restricted, HEL specific T hybridoma cells (3B11.1 or A2.A2) or with transgenic T cells expressing I-A^k-restricted, HEL specific TCR (3A9). T cell activation was measured by IL-2 release. The T cell hybridomas (figure 4A) or transgenic T cells (figure 4B) were activated only if the donor tumor cells were I-A^k positive and DC were present, or if intact, live I-A^k-transfected tumor cells were used as controls (data not shown). Therefore, tumor vaccine derived antigens and MHC class II acquired by DC activate tumor-specific $CD4^+$ T cells restricted to the genotype of the tumor.

MHC II and tumor antigen/peptide could be transferred as pre-formed complexes or transferred MHC II may assemble in the DC with transferred tumor antigen or with other antigen acquired by the DC. If MHC II/peptide complexes are pre-formed in the tumor cells, then DC exposed to HEL after MHC II transfer will not form MHC II-peptide complexes and will not activate $CD4^+$ T cells. Conversely, if the MHC II-peptide complexes form in the recipient DC after MHC II transfer, then DC containing transferred MHC II and pulsed with antigen will activate $CD4^+$ T cells. To test these alternatives, M12.C3.F6 cells were pulsed overnight with intact HEL protein to allow for the formation of $I\text{-}A^k$-HEL complexes. Control M12.C3.F6 cells were not incubated with HEL. M12.C3.F6 cells were then freeze-thaw lysed and incubated with FVB DC. Following extensive washing to remove residual tumor material, recipient DC were cultured with the T cell hybridoma A2.A2 in the presence or absence of exogenous HEL protein. As is shown in figure 4C, $CD4^+$ T cells are activated only if $I\text{-}A^k$ and HEL are present in the tumor cells prior to transfer. DC that were fed exogenous HEL after the transfer of $I\text{-}A^k$ did not activate T cells. These data demonstrate that MHC class II – peptide complexes form in the tumor cells and transfer to DC to stimulate $CD4^+$ T cells.

Tumor MHC class II expression and DC are required for maximum in vivo $CD4^+$ T cell activation

Previous in vivo studies have demonstrated enhanced activation of tumor-specific $CD4^+$ T cells in response to MHC II vaccines. If p-MHC transfer from tumor cells to DC is required for in vivo induction of tumor immunity, then MHC II vaccine enhanced $CD4^+$ T cell activation would be dependent on the presence of DC. Conversely, if

pMHC transfer did not occur or was irrelevant, then T cell activation would require neither tumor cell MHC class II expression nor the presence of DC. To address this question, we mock treated or DC depleted mice prior to tumor challenge with SaI/HEL or SaI/A^k/HEL cells. One week later, $CD4^+$ T cells were isolated from animals and tested for their response to HEL presented by an I-A^k B cell lymphoma, M12.C3.F6. Maximum activation of HEL-specific $CD4^+$ T cells occurred when the immunizing tumor cells expressed MHC class II and DC were present, although there was some $CD4^+$ T cell activation when mice were immunized with MHC II negative tumor cells (figure 5). Therefore cross-priming results in some T cell activation; however, maximum $CD4^+$ T cell activation requires DC and MHC II expression by vaccine cells, supporting the hypothesis that pMHC II complexes generated by tumor cells are acquired by DC for T cell activation.

Discussion

We have concluded from previous experiments that MHC II vaccine cells are the principal APC in vivo and that they activate tumor-specific $CD4^+$ T cells by directly presenting endogenously synthesized tumor antigens. This conclusion was based on experiments in which activated tumor-specific $CD4^+$ T cells were MHC restricted to the class II genotype of the vaccine cells, and not to the genotype of host DC (15, 16). The findings reported here confirm the genotype restriction of activated $CD4^+$ T cells, but suggest that the process of activation is more complex than we originally proposed. The requirement for DC coupled with the transfer of pMHC complexes from the vaccine cells to DC, makes it likely that DC are the primary APC and that they activate $CD4^+$ T cells by presenting pMHC complexes acquired from MHC II vaccine cells. Yewdell and Haeryfar have speculated that this mechanism of antigen transfer occurs during the activation of $CD8^+$ T cells and have coined the term "cross-dressing" to describe it (38). The results presented here validate the concept of cross-dressing and demonstrate that it occurs during the activation of $CD4^+$ T cells by MHC II cancer vaccines.

Dendritic cells are considered the most efficient APC (22). Their primary mechanism for acquiring soluble protein antigen is by endocytosis, although they also acquire antigen through exosome uptake (39-41). In both processes, DC degrade acquired antigen to peptides and load the resulting peptides onto DC-encoded MHC class I and II molecules for presentation at their cell surface. Because the antigenic epitopes presented by DC are not synthesized by the DC, these mechanisms of antigen uptake and presentation are called "cross-presentation". Cross-presentation was first described by Bevan (42) and is well accepted as a mechanism for initiating T cell responses (21, 43).

The studies presented here identify acquisition of peptide-MHC complexes by DC as a potential additional process for activating T cells. Although DC are the APC in both cross-presentation and cross-dressing, the two processes have fundamental differences. In cross-dressing, donor cells synthesize and generate both the antigenic peptides and MHC molecules that are subsequently presented by DC. In contrast, during cross-presentation antigen is synthesized by donor cells and acquired by DC which then process and load the peptides onto DC-synthesized MHC proteins. Therefore, although DC actively generate peptide-MHC complexes in cross-presentation, their role in cross-dressing is more passive and is limited to presenting MHC-peptide complexes acquired from donor cells.

The transfer of MHC molecules from donor to recipient DC has been observed previously. For example, T cell responses against transferred allogeneic MHC molecules have been reported (35, 44, 45), and the transfer of exosomes containing donor cell MHC molecules is well established (40, 46, 47). The present report establishes that antigenic peptides are an integral component of the transferred MHC molecules and that T cells are activated to the intact transferred complexes. These data contribute to the growing evidence that the transfer of macromolecules from donor cells to DC is an important mechanism for priming T cells.

Figure 6 is a schematic model of how MHC II vaccines may activate $CD4^+$ T cells by cross-dressing. Initial inoculation of vaccine cells induces local inflammation which causes vaccine cell necrosis and persistent low-grade inflammation at the inoculation site. The necrotic vaccine cells release peptide-MHC complexes, which are picked up by DC and without further processing are presented on the DC's plasma membranes. As the

"cross-dressed" DC mature in response to the local pro-inflammatory signals, they become activated, express increasing levels of costimulatory molecules, and migrate to the draining lymph nodes. In the lymph nodes the activated DC present the pMHC complexes plus costimulatory signals, and T cells with the appropriate TCR are primed and activated. Cross-presentation of tumor antigens by DC is also likely to occur; however, by itself it is not sufficient for induction of optimal tumor immunity. This model is consistent with earlier observations that activated T cells are tumor-specific and restricted to the MHC genotype of the vaccine cells, and explains why vaccine cell expression of MHC II molecules is essential (15, 33). This model is also consistent with the activation of T cells in the lymph nodes and explains why MHC II vaccine cells are not found in lymph nodes after vaccination.

Cross-dressing of DC may enhance MHC II vaccine efficacy because peptides in the transferred complexes are novel tumor antigen epitopes to which the host is not tolerant. Individuals with progressively growing tumors are frequently tolerant to the antigens of their tumors (48-50). However, tumor-bearing individuals may not be tolerant to tumor antigen epitopes generated by MHC II vaccines, because the vaccine cells may process and present novel peptides that are not generated by DC. Professional APC such as DC, co-express MHC class II, Ii, and DM. Expression of Ii favors the presentation of exogenously acquired antigens (51). However, MHC II vaccine cells do not express Ii, so their peptide repertoire is skewed towards peptides derived from endogenous sources. DM influences the peptide repertoire by editing peptides as they bind to MHC II molecules in endosomal compartments, and genetically manipulated APC that do not express Ii and DM produce a different repertoire of peptides than that

presented by DM positive cells (52). Therefore, DC carrying pMHC complexes acquired from MHC II vaccine cells may present novel tumor antigen epitopes and prime an expanded repertoire of tumor-specific $CD4^+$ T cells.

Although it is unclear if cross-dressing occurs in other types of immunizations, the transfer of novel pMHC complexes from MHC $II^+Ii^-DM^-$ MHC II vaccine cells to DC may be an important step in activating $CD4^+$ T cells that facilitate tumor immunity. Further studies are necessary to determine to what extent cross-dressed DC activate T cells, which subset of DC are most important for cross-dressing, and what role, if any, cross-dressing has in tolerance induction.

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Table I. Vaccine efficacy provided by MHC class II vaccines requires host DC. CD11c-DTR or non-transgenic littermates were given a single dose of diphtheria toxin (DTx) or left untreated 6 hours prior to tumor challenge. Mice were monitored daily for ascites tumor growth and sacrificed when they became moribund. Tumor incidence is the number of mice who became moribund by day 20 divided by the total number of mice challenged.

CD11c-DTR	**DTx**	**Tumor Challenge**	**Tumor Incidence**
-	-	SaI	5/5
+	-	SaI	4/5
-	+	SaI/A^k	1/7
+	-	SaI/A^k	1/9
+	+	SaI/A^k	7/8

Figure 1. MHC class II complexes transfer from dead tumor cells to DC. (A) Splenic DC from FVB mice (*H-2*^{q}) were co-cultured with live or freeze-thawed SaI or SaI/A^{k} cells for three hours and adherent DC were washed extensively to remove tumor material. DC were then stained for CD11c and I-A^{k} and analyzed by flow cytometry. (B) Splenic DC can be resolved from tumor cells by forward scatter and side scatter and are PI negative. All of the DC flow cytometry data presented used the DC bitmap shown in the upper panels. (C) Transferred MHC II is stable on recipient DC. Splenic DC from FVB mice were co-cultured with freeze-thawed SaI/A^{k} cells and adherent cells were washed to remove tumor material, and incubated in media containing GM-CSF and IL-4 for 0, 24, or 48 hours. DC were then analyzed for I-A^{k} expression. (D) DC mature during culture. Cells from (C) were stained for CD80 and CD86 at 0 hours (open histograms) or 20 hours (shaded histograms). (E) MHC class II transfers from dead tumor cells to bone marrow dendritic cells. BMDC were generated from the bone marrow of FVB mice, co-cultured with freeze-thawed SaI or SaI/A^{k} cells as in (A), and analyzed for I-A^{k} expression. These data are from one of three or more independent experiments.

Figure 2. MHC class I and II molecules transfer from a variety of tumor cells to DC. (A) Splenic DC from FVB mice were co-cultured with freeze-thawed lysates of B16.BL6/A^{b}, B16 MELF10/A^{b}, A20, or M12.C3.F6 cells as in figure 1A and analyzed by flow cytometry for I-A^{b} (B16 lines), I-A^{d} (A20) , or I-A^{k} (M12.C3.F6) expression. (B) Splenic DC from FVB mice were co-cultured with freeze-thaw lysates of the indicated tumor cells and subsequently analyzed for K^{b} (from 3LL), D^{d} (from 4T1), and K^{k} (from

SaI) expression. These data are from two or more independent experiments for each cell line.

Figure 3. MHC II and lipid transfer from tumor cells to DC requires cell-cell contact and DC-expressed plasma membrane receptors. (A) Splenic DC from FVB mice and freeze-thawed SaI/A^k lysate were cultured in the lower and upper chambers, respectively, of a transwell separated by an 8μm semi-permeable membrane. After three hours of culture, DC were stained for CD11c and I-A^k expression and analyzed by flow cytometry. (B) MHC II transfer requires DC-expressed plasma membrane receptors. CFSE-labeled splenic DC from FVB mice were treated with pronase prior to their incubation with freeze-thawed SaI/A^k lysate. These data are from one of three independent experiments. (C) Plasma membrane lipids are transferred from tumor cells to DC. Splenic DC from FVB mice were incubated with freeze-thawed SaI or SaI/A^k cells that were labeled with the lipophilic dye, DiD, processed as in figure 2A, and examined by scanning laser confocal microscopy. (D) Transferred MHC II is retained on recipient DC but becomes dispersed with time. Splenic DC from FVB mice were incubated with freeze-thawed SaI/A^k cell lysates for 3 hours, separated from tumor material, and cultured an additional 48 hours in the absence of tumor lysates. DC were then stained for CD11c and I-A^k and analyzed by confocal microscopy. A minimum of 25 cells was examined for each cell type.

Figure 4. Complexes of MHC class II and tumor encoded peptides transfer from tumor cells to DC and activate $CD4^+$ T cells. FVB splenic DC were incubated with freeze-

thawed SaI/HEL or SaI/A^k/HEL cells as in Figure 1A and used as APC with the I-A^k-restricted, HEL-specific T hybridoma 3B11.1 (A) or 3A9 transgenic T cells (B). (C) Transferred MHC II complexes contain tumor-generated peptides. I-A^k-restricted, HEL-specific hybridoma cells (A2.A2) were cultured in the presence or absence of exogenous HEL, with freeze-thawed M12.C3.F6 cells that were loaded with exogenous HEL prior to freeze-thaw. IL-2 release was quantified by ELISA. Error bars are the standard deviation of the mean. These data are from one of three independent experiments (panels A and B) or two independent experiments (panel C).

Figure 5. In vivo $CD4^+$ T cell activation requires both DC and MHC class II expression by tumor cells. DTx treated or untreated CD11c-DTR mice were immunized with 8×10^5 SaI/HEL or SaI/A^k/HEL cells on day 0, and their splenic $CD4^+$ T cells isolated on day 6 and co-cultured with I-A^k-positive APC (M12.C3.F6 B lymphoma cells) and exogenous HEL. Supernatants were harvested on day 7 and assayed for IL-2 release by ELISA. Error bars are the standard deviation of the mean. These data are from one of two independent experiments. * indicates statistical significance ($p \leq 0.05$) from other treatments.

Figure 6. Schematic model for the activation of tumor-specific T cells by MHC class II vaccines. MHC II vaccine cells contain MHC complexes loaded with tumor-generated peptides. At the site of injection, the vaccine cells trigger an inflammatory response that causes the release of plasma membrane fragments containing peptide-MHC complexes. Immature DC acquire the peptide-MHC complexes and concurrently receive maturation

signals from the inflammatory milieu. DC mature and migrate to the draining lymph nodes where they activate tumor-specific T cells.

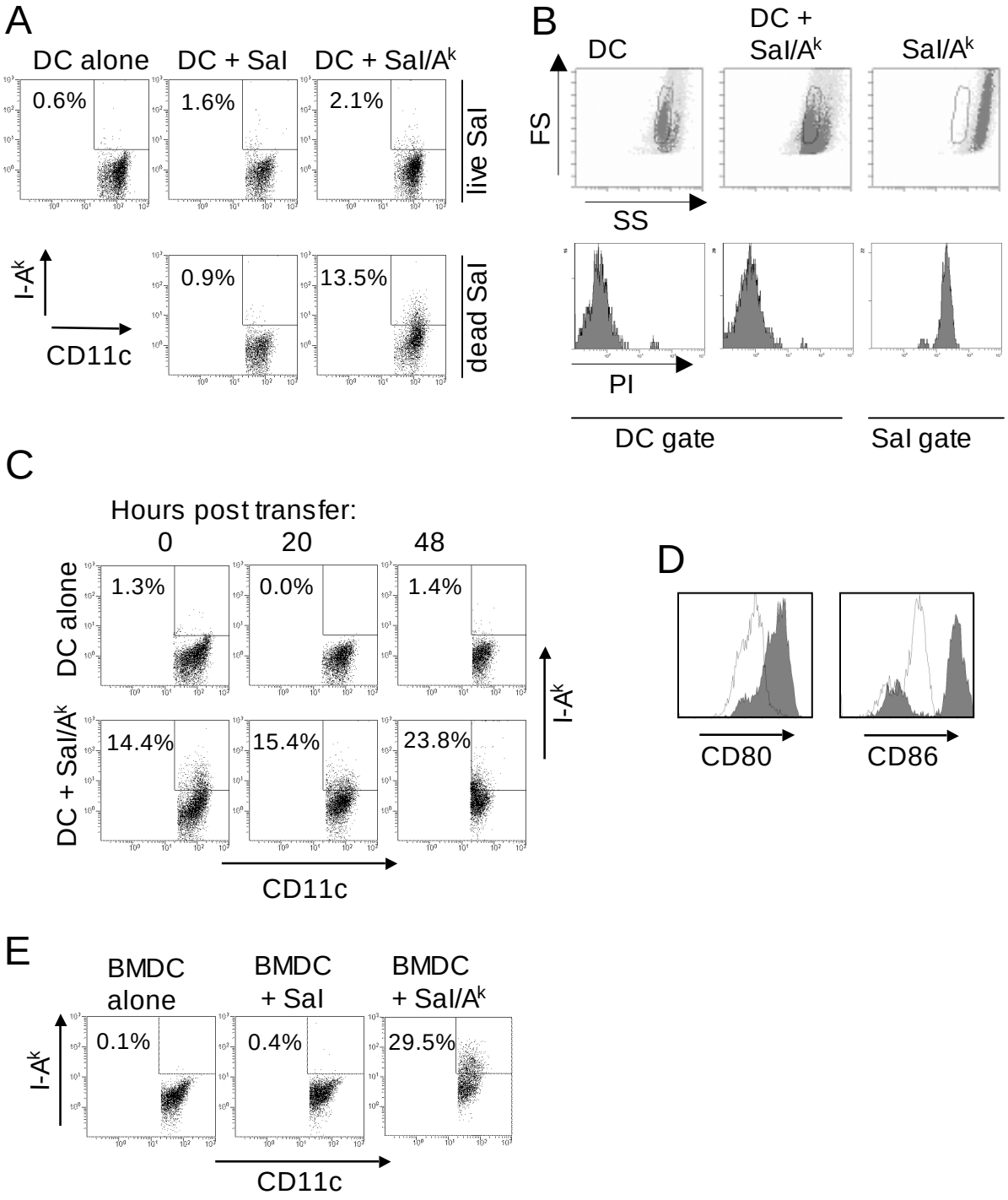
A
DC alone
DC + SaI
DC + SaI/A^k
0.6%
1.6%
2.1%
live SaI
I-A^k
CD11c
0.9%
13.5%
dead SaI
B
DC
DC + SaI/A^k
SaI/A^k
FS
SS
PI
DC gate
SaI gate
C
Hours post transfer:
0
20
48
DC alone
1.3%
0.0%
1.4%
DC + SaI/A^k
14.4%
15.4%
23.8%
I-A^k
CD11c
D
CD80
CD86
E
BMDC alone
BMDC + SaI
BMDC + SaI/A^k
I-A^k
0.1%
0.4%
29.5%
CD11c

Figure 1

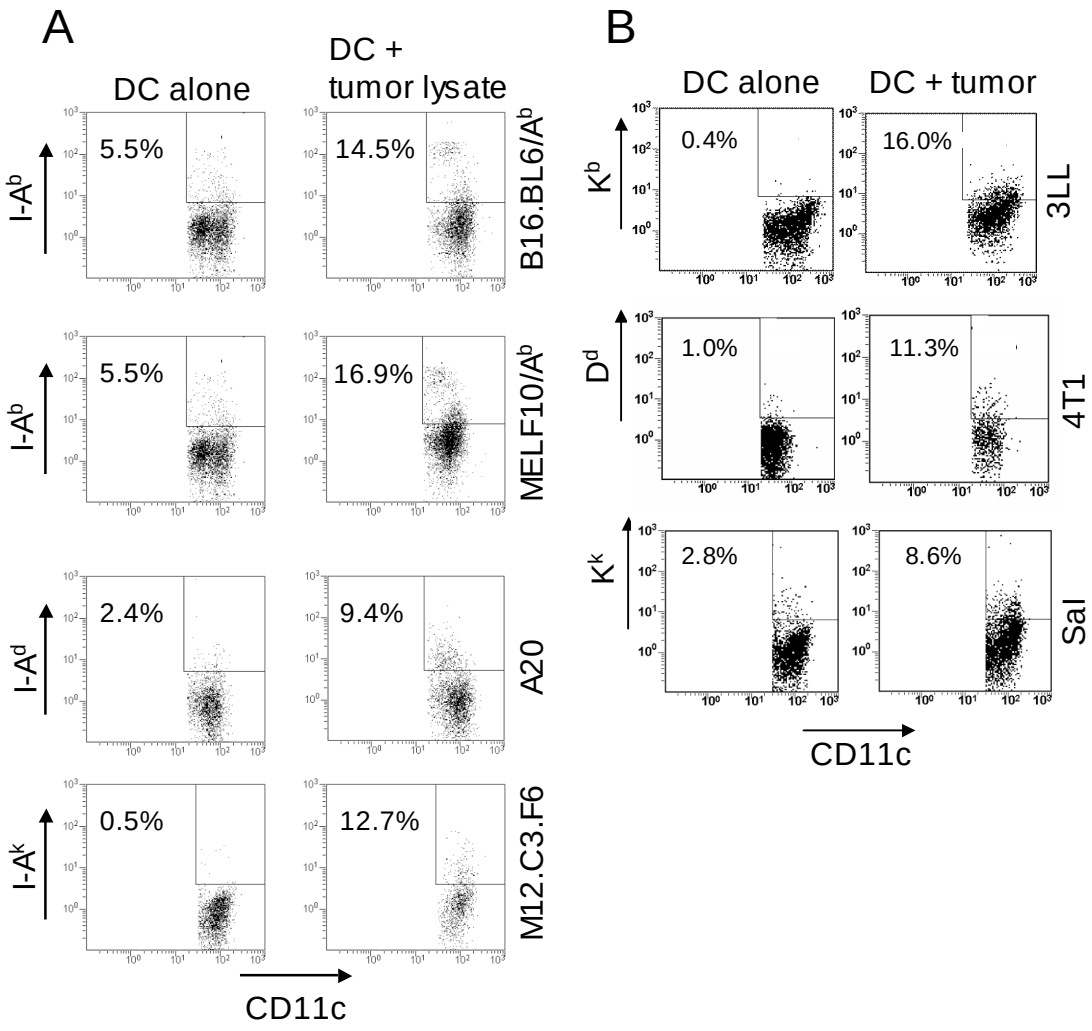
A
DC alone
DC +
tumor lysate
5.5%
14.5%
B16.BL6/Ab
I-Ab
5.5%
16.9%
MELF10/Ab
I-Ab
2.4%
9.4%
A20
I-Ad
0.5%
12.7%
M12.C3.F6
I-Ak
CD11c
B
DC alone
DC + tumor
0.4%
16.0%
3LL
Kb
1.0%
11.3%
4T1
Dd
2.8%
8.6%
Sal
Kk
CD11c

Figure 2

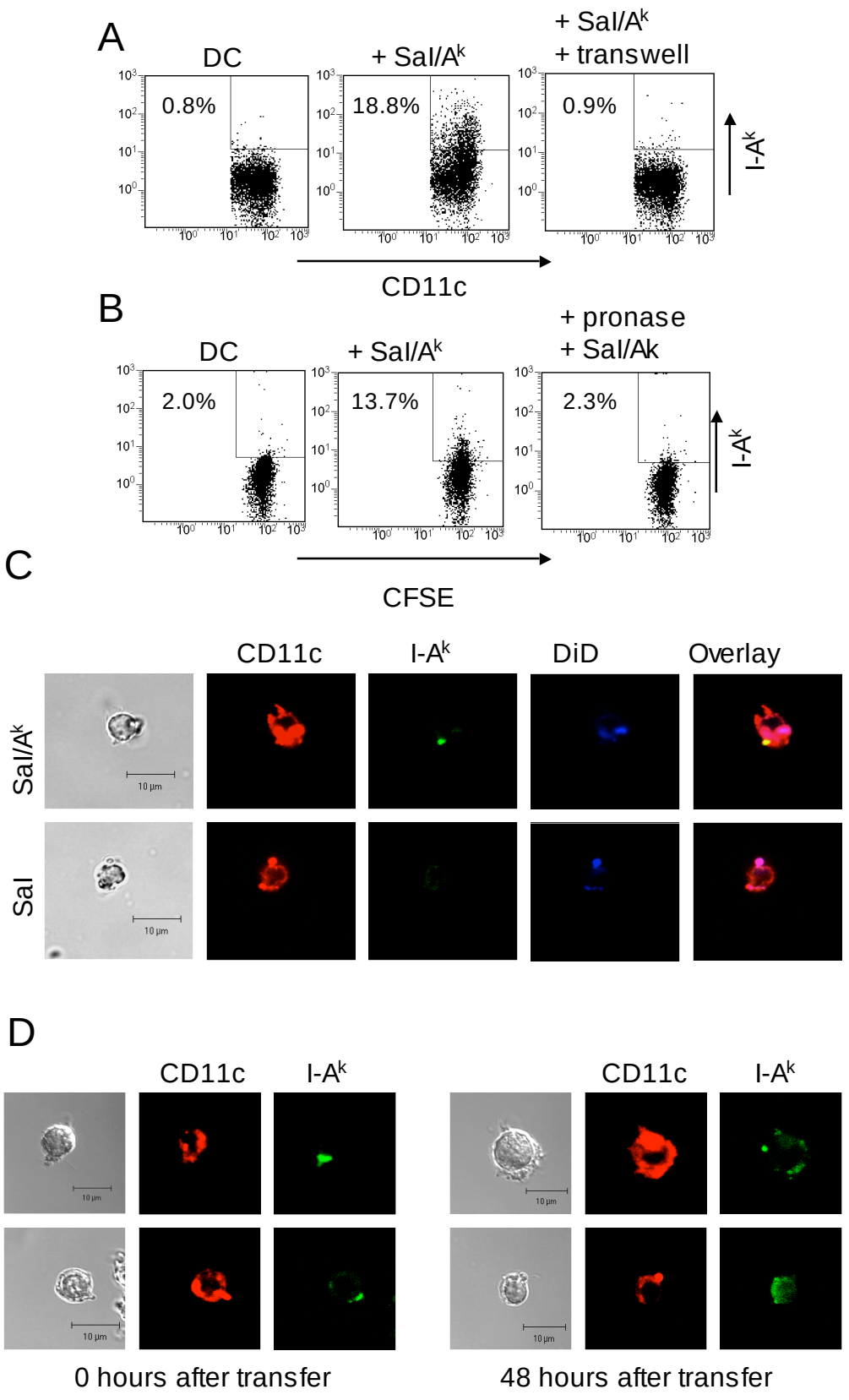
A
DC
+ SaI/Ak
+ SaI/Ak
+ transwell
0.8%
18.8%
0.9%
I-Ak
CD11c
B
DC
+ SaI/Ak
+ pronase
+ SaI/Ak
2.0%
13.7%
2.3%
I-Ak
CFSE
C
CD11c
I-Ak
DiD
Overlay
SaI/Ak
SaI
10 µm
D
CD11c
I-Ak
CD11c
I-Ak
10 µm
0 hours after transfer
48 hours after transfer

Figure 3

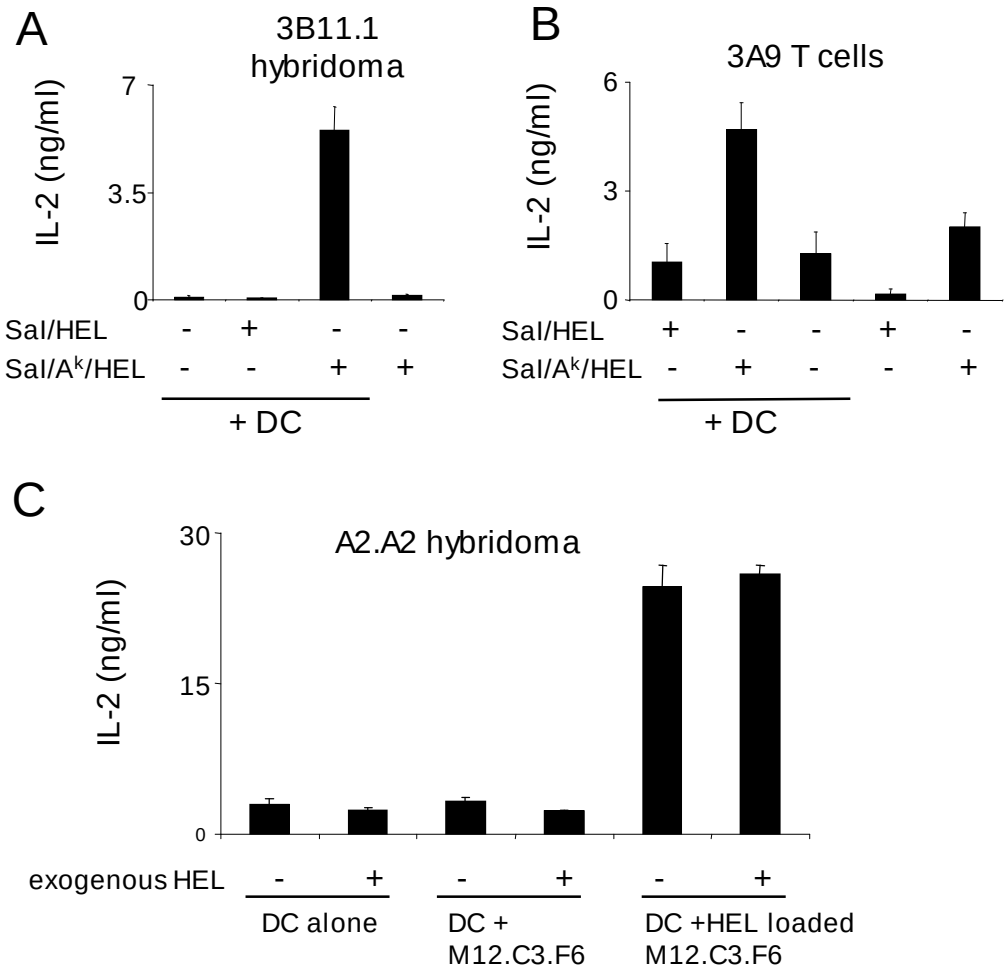
A
3B11.1 hybridoma
IL-2 (ng/ml)
7
3.5
0
SaI/HEL - + - -
SaI/Ak/HEL - - + +
+ DC
B
3A9 T cells
IL-2 (ng/ml)
6
3
0
SaI/HEL + - - + -
SaI/Ak/HEL - + - - +
+ DC
C
A2.A2 hybridoma
IL-2 (ng/ml)
30
15
0
exogenous HEL - + - + - +
DC alone
DC + M12.C3.F6
DC +HEL loaded M12.C3.F6

Figure 4

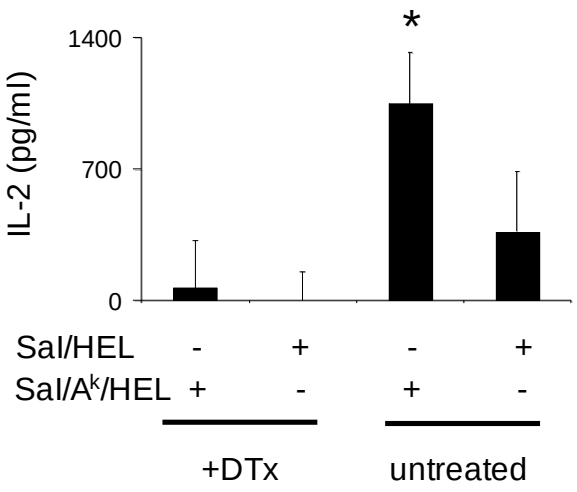

Figure 5

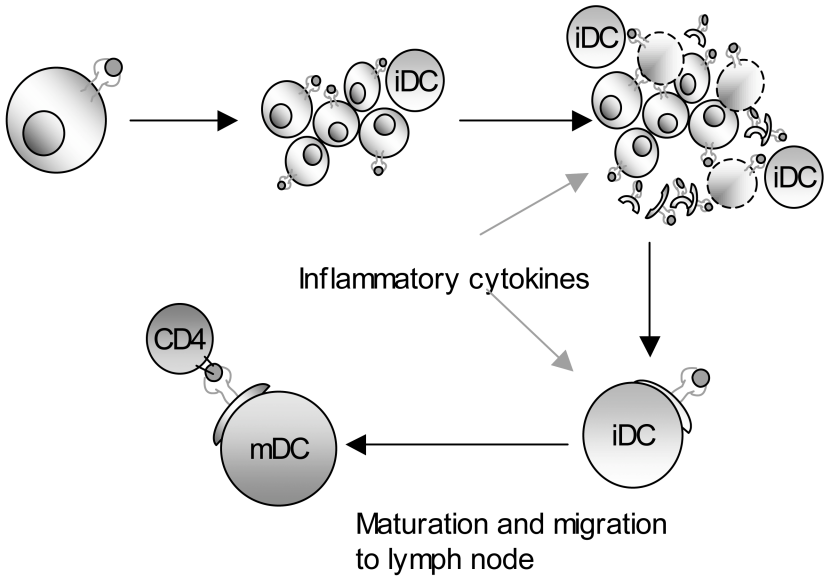
iDC
iDC
iDC
Inflammatory cytokines
CD4
mDC
iDC
Maturation and migration
to lymph node

Figure 6

Appendix B

Dendritic Cells Cross-Dressed with Peptide MHC Class I Complexes Prime $CD8^+$ T Cells[1]

By

Brian P. Dolan, Kenneth D. Gibbs, Jr., and Suzanne Ostrand-Rosenberg[2]

Department of Biological Sciences

University of Maryland Baltimore County

Baltimore, MD 21250

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Footnotes

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[2] Address correspondence and reprint requests to Dr. S. Ostrand-Rosenberg, Department of Biological Sciences, U. of Maryland, 1000 Hilltop Circle, Baltimore, MD 21250.

[3] Abbreviations used in this study:

BMDC, bone marrow-derived dendritic cell

DC, dendritic cell

DTR, diphtheria toxin receptor

DTx, diphtheria toxin

Abstract

The activation of naïve $CD8^+$ T cells has been attributed to two mechanisms: cross-priming and direct priming. Cross-priming and direct priming differ in the source of antigen and in the cell that presents the antigen to the responding $CD8^+$ T cells. In cross-priming, exogenous antigen is acquired by professional antigen presenting cells, such as dendritic cells (DC), which process the antigen into peptides that are subsequently presented. In direct priming, the antigen presenting cells, which may or may not be DC, synthesize and process the antigen and present it themselves to $CD8^+$ T cells. Here we demonstrate that naïve $CD8^+$ T cells are activated by a third mechanism, called "cross-dressing." In cross-dressing, DC directly acquire MHC class I-peptide complexes from dead, but not live, donor cells by a cell-contact-mediated mechanism, and present the intact complexes to naïve $CD8^+$ T cells. Such DC are "cross-dressed" because they are "wearing" peptide-MHC complexes generated by other cells. $CD8^+$ T cells activated by cross-dressing are restricted to the MHC class I genotype of the donor cells and are specific for peptides generated by the donor cells. In vivo studies demonstrate that optimal priming of $CD8^+$ T cells requires both cross-priming and cross-dressing. Thus, cross-dressing may be an important mechanism by which DC prime naïve $CD8^+$ T cells and may explain how $CD8^+$ T cells are primed to antigens which are inefficiently cross-presented.

Introduction

Antigen presentation to $CD8^+$ T lymphocytes is postulated to occur through two mechanisms, which differ from each other by the type of cell that processes and presents the peptide antigen. According to the direct antigen presentation mechanism, $CD8^+$ T cells recognize their cognate peptide-MHC class I complexes on the surface of the cells that synthesized the antigen, such as a virally-infected cells or malignant cells (1). Alternatively, $CD8^+$ T cells recognize peptide-MHC class I complexes on the surface of cells, which have captured exogenously synthesized antigen, and subsequently process and present the antigen bound to their own MHC class I proteins. This latter mechanism is termed cross-presentation and dendritic cells[3] are the predominant cell population that captures and presents antigen (2, 3). If the process of cross-presentation results in the activation of naïve T cells, then the T cells have been activated by "cross-priming."

Although compelling evidence supports cross-priming as the principal mechanism for activating naïve $CD8^+$ T cells (2, 4, 5), other studies are consistent with $CD8^+$ T cells being activated by the direct presentation mechanism (6, 7). The concept of cross-priming is supported by many reports demonstrating that $CD8^+$ T cells specific for virally-infected cells are primed by DC cross-presenting virally-derived peptides (8-11), although virally-infected DC also directly present viral antigens to $CD8^+$ T cells (12, 13). Ultimately, $CD8^+$ T cell priming to viral infections may be the result of both cross-priming and direct priming (14). Cross-priming has also been demonstrated for $CD8^+$ T cells activated to tumor antigens (15-17); however, other studies suggest that priming of tumor-specific $CD8^+$ T cells occurs through the direct presentation of tumor antigens by

tumor cells (18, 19). Therefore, a consensus model for $CD8^+$ T cell priming does not exist.

Here we report that $CD8^+$ T cells can be primed by a third mechanism, termed "cross-dressing." During cross-dressing, peptide-MHC class I complexes are transferred to DC from dead donor cells that synthesized the complexes. Recipient DC then use the acquired peptide-MHC class I complexes to activate $CD8^+$ T cells that are peptide-specific and restricted to the MHC class I genotype of the donor cells. Optimal priming of $CD8^+$ T cells probably results from a combination of cross-dressing and cross-priming, because cross-dressing efficiently activates DC that are not efficiently activated by cross-priming.

Materials and Methods

Mice

Breeding stocks of BALB/c, C57BL/6, FVB, OT-I, and CD11c-DTR (Itgax-DTR/EGFP) mice on a BALB/c background were from The Jackson Laboratory (Bar Harbor ME). MHC I/II$^{-/-}$ mice and OT-I/RAG 2$^{-/-}$ mice were from Taconic (Germantown NY). CD11c-DTR mice were screened as described (20). OT-I mice were identified by immunofluorescent staining of peripheral blood lymphocytes for CD8 and $V_{\alpha}2$. H-2^{b}/CD11c-DTR$^{+/-}$ mice were generated by crossing CD11c-DTR mice with MHC class I/II deficient mice and the CD11c-DTR$^{+/-}$ F1 offspring were back-crossed to MHC class I/II-deficient mice. CD11c-DTR$^{+/-}$ F2 mice that were negative for both MHC class I and II (identified by immunofluorescence staining for the absence of peripheral CD4^{+} and CD8^{+} T cells) were then crossed with C57BL/6 mice. The F1 mice of this cross were subsequently used in experiments. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Cells, transfections, and antibodies

B16.BL6 8.2, A20, EL4 and EL4/ova were cultured as described (20-22). NIH3T3 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan UT). DAP/ova cells (23) were maintained in RPMI (Biofluids, Rockville MD) supplemented with 10% FCS , 1mM HEPES (Life Technologies, Rockville MD), 5 x 10^{-6} M beta-mercaptoethanol (Sigma, St. Louis MO), 400 μg/ml G418 (CalBiochem, LaJolla CA), and 200 μg/ml zeocin (Invitrogen, Carlsbad CA). Media for all cell lines contained 1% penicillin, 1% streptomycin, 1% gentamicin

(Biofluids), and 1% Glutamax (Life Technologies) ("complete" medium). The DAP/ova/K^b cell line was generated by transfection using Lipofectamine Plus (Invitrogen) according to the manufacturer's recommendations. Approximately 4 μg of the K^b plasmid pKbT.8 (24, 25) were linearized with Pvu I (Fermentas Life Sciences, Hanover MD), ethanol precipitated, resuspended in 100 μl of DMEM (Biofluids), mixed with 16 μl of Plus reagent, and incubated for 15 minutes at room temperature. One hundred μl of DMEM containing 10 μl of Lipofectamine was then added, and the mixture incubated for an additional 15 minutes. The DNA mixture was then added to DAP/ova cells plated the previous night at 4 x 10^5 cells/well of a 6 well plate, in a final volume of 0.4 ml DMEM. Cells were cultured with DNA for 3 hours before the addition of 2 ml complete media and an additional 60 μl FCS. H-2K^{b+} cells were identified by staining with fluorescently tagged mAb (clone AF6-88.5, BD Pharmingen San Diego, CA). Bulk H-2K^{b+} transfectants were fluorescently sorted using a Coulter Epics Altra cell sorter. Fibroblasts were generated as follows: minced tail tissue was resuspended in 2.5 ml of a 2mg/ml collagenase IV (Worthington, Lakewood, NJ) solution supplemented with 10 μl of elastase (MP Biomedicals, Aurora OH), shaken gently for 1-2 days at 4°C, washed with PBS, and adherent cells cultured in complete IMDM supplemented with 10% FCS. The following antibodies directly coupled to FITC or PE were purchased from BD Pharmingen: CD11c (HL3), CD8 (53-6.7), CD4 (GK1.5), CD69 (H1.2F3), K^k (36-7-5), K^b (AF6-88.5), D^d (34-2-12S), and $V_\alpha 2$ (B20.1). Anti-ovalbumin (OVA) mAb antibody (clone 14) and anti-β-tubulin mAb (Tub 2.1) were from Sigma. Sheep anti-mouse-HRP antibody was from Amersham.

Western Blotting

Western blots for β-tubulin were preformed as described (26). Immunoblotting for OVA followed the same experimental procedure except the primary antibody for OVA (mAb clone 14) was diluted 1:5000 in 0.5% Tween-20-TBS solution containing 0.5% non-fat milk.

DC isolation and culture

Splenic DC were isolated and cultured as described (20) using CD11c microbeads (Miltenyi Biotech, Auburn CA). Bone marrow derived-DC (BMDC) were isolated by flushing the bone marrow from femurs of 3-6 month old mice followed by red blood cell depletion using ACK lysing buffer (Biofluids). Cells were cultured in DC medium (complete RPMI supplemented with 5% FCS, 1mM HEPES, 5 x 10^{-6} M beta-mercaptoethanol, 20 ng/ml GM-CSF and 10 ng/ml IL-4 (RDI, Flanders NJ)). Medium was changed every two days and cells were 80-90% $CD11c^{+}$ by day 6. DC were matured by overnight incubation in DC media supplemented with 2.5 μg/ml LPS (Sigma) and 1 μg/ml CD40 mAb (clone 3/23 from BD Pharmingen).

MHC I transfer experiments

DC (either splenic or BM derived) were plated in 1.0 – 1.5 ml BMDC media without GM-CSF and IL-4 at 1-2 x 10^{6} cells/well in 6 well plates. Donor cells were killed by resuspending to a concentration of 10^{7}/ml in RPMI without serum and freezing at -80° C for 15-30 minutes followed by rapid thawing in a 37° C water bath. Cells were freeze-thawed for 1-3 cycles until 100% of the cells were trypan blue positive. Apoptosis was

induced by culturing donor cells in 20 μm camptothecin (27) (Sigma) for three days, after which ~75% of the cells were trypan blue positive. Dead donor cells were added to DC at 3-5x cell equivalents. After 3 hours of culture, the mostly attached splenic DC were washed extensively in warm PBS to remove dead material, and the DC were removed by cell-scraping. For transfer to BMDC, dead donor cells and live DC were centrifuged at 1100g through Ficoll-Paque (Pharmicia-Biotech, Uppsala Sweden) for 15 minutes at room temperature, and the live BMDC were recovered at the Ficoll-media interface. If dead cells remained, the process was repeated until BMDC contained <5% dead cells as measured by trypan blue uptake. Where indicated, donor cells were cultured with 200 units/ml IFN-γ (Pierce Endogen, Rockford IL) for two days prior to freeze-thawing. In some experiments, freeze-thawed cells were centrifuged at 300g and the resulting supernatant used for MHC transfer. In other experiments, DC were plated in the lower half of an 8.0 μm transwell (Corning, Corning NY) and freeze-thawed cells added to the top half. In control experiments, 10 μl of 50 nm FITC-coated latex beads (Polyscience, Inc., Warrington, PA) were added to the top half containing the freeze-thawed cells.

Antigen Presentation Assays

Splenic DC were plated in 96 well plates in DC medium at 10^5 cells/well. One hour later freeze-thawed donor cells at 3-4x cell equivalents or soluble OVA protein (Sigma) were added. Following a three hour incubation, wells were washed with excess PBS to remove dead material. BMDC were incubated with freeze-thawed donor cells or with soluble OVA protein for three hours, the mixture passaged over ficoll to remove dead material, and the purified BMDC plated at 10^5 cells/well in 96 well-plates. $CD8^+$ OT-I T cells

were isolated using $CD8^+$ T cell isolation kits according to the manufacturer's suggested protocol (Miltenyi Biotech), and added to the purified BMDC at 10^5 cells/well for a total volume of 200 μl of complete RPMI medium/well. OT-I activation was quantified by assaying culture supernatants for IL-2 and IFN-γ by ELISA (Pierce Endogen) as described (28), or by double staining for CD69 and $V_\alpha 2$.

In vivo T cell proliferation

OT-I T cells were isolated by magnetic bead sorting from OT-I/RAG $2^{-/-}$ mice and incubated for 10 minutes at room temperature in 2.5 μM CFSE (Molecular Probes, Eugene OR) in PBS at 10^7 cells/ml. The reaction was quenched by the addition of 1 ml FCS and the cells were washed with excess PBS. 5 x 10^6 labeled cells were injected i.v. into the tail vein of recipient mice and 8-24 hours later mice were inoculated s.c. in the lateral, lower abdominal region with 5-10 x 10^6 live, freeze-thawed, or a 1:1 mixture of live and freeze-thawed cells. Three days later mice were sacrificed, the draining lymph node (superficial inguinal) removed and dissociated, and the cells were examined by flow cytometry for dilution of CFSE. Background auto fluorescence was determined using mice which did not receive CFSE-labeled cells and was gated out. The proliferation index was calculated by dividing the total number of $CFSE^+$ cells in all divisions by the number of parental cells originally present and was calculated using ModFit software (Verity Software House) (29). In CD11c-depletion experiments, (C57BL/6 x CD11c-DTR) F1 mice were injected i.p.with 3 ng/g body weight diphtheria toxin (DTx, Sigma), and six hours later, inoculated with live and/or freeze-thawed donor cells.

Statistical Analysis

Standard deviations and Student's T test were calculated using Microsoft Excel 2002.

Results

MHC class I molecules transfer from dead cells to DC

Acquisition of MHC class I by DC was first assessed in vitro using bone marrow-derived DC (BMDC) and allogeneic donor cells. BMDC from FVB (*H-2*^{q}) mice were incubated for three hours with freeze-thawed genetically mis-matched DAP/ova (*H-2*^{k}) fibroblasts, and the resulting cells were purified by passage over a ficoll gradient. Ficoll purified cells (figure 1A, top left panel) bitmap to a different location than freeze-thawed cells (figure 1A, bottom left panel), are >93% viable (figure 1A, middle panel), and are > 90% CD11c$^+$ (right panel), identifying them as DC. To determine if MHC class I transfers to DC, purified cells were stained for donor genotype H-2K^k molecules. As seen in figure 1B (top left panel), *H-2*^{q} DC incubated with freeze-thawed DAP/ova cells stain for H-2K^k, demonstrating that the DC have acquired MHC class I molecules. Similar results were obtained if MHC class I-deficient BMDC were used as recipient DC instead of FVB BMDC (data not shown). MHC class I molecules also transferred to DC from apoptotic DAP/ova cells (data no shown). In agreement with previous studies of MHC class II transfer to DC (20), MHC molecules are not detected on BMDC cultured with live DAP/ova cells (figure 1B bottom left panel).

To test if MHC class I molecules were retained during DC maturation, DC were co-cultured with freeze-thawed DAP/ova cells, purified by ficoll gradient, and cultured overnight in the presence of LPS and mAbs to CD40 to induce maturation. The following day, BMDC were stained for donor MHC class I (*H-2K*^{k}). As shown in figure

1B (bottom right panel), donor H-2K^k protein is detected after overnight culture and DC maturation, demonstrating the stability of transferred MHC class I molecules.

To ensure specificity of MHC class I transfer, K^b-expressing DAP/ova cells were generated (DAP/ova/K^b; figure 1C), and we compared H-2K^b transfer to H-2^q DC from freeze-thawed DAP/ova/K^b vs. DAP/ova cells. H-2K^b was detected on FVB BMDC exposed to DAP/ova/K^b cells, but not on BMDC exposed to the parental DAP/ova cells (figure 1D), confirming the transfer of donor MHC class I molecules to DC. Collectively, these results demonstrate that DC acquire MHC class I molecules from dead donor cells.

MHC class I transfer requires cell-to-cell contact

To determine if the particulate material or the supernatant of the freeze-thawed cells was involved in MHC class I transfer, freeze-thawed DAP/ova cells were centrifuged at 300g for three minutes, and the supernatant removed and added to cultures of FVB BMDC. Three hours later the DC were purified and labeled for H-2K^k. As shown in figure 1E, MHC class I transfer in the presence of the supernatant is ~30% of the level obtained with non-fractionated freeze-thawed donor cells. To test if cell contact between dead cells and DC is required for MHC class I transfer, FVB BMDC were separated from freeze-thawed DAP/ova cells for the three hour culture period using an 8.0 μm transwell, and then stained for H-2K^k. Under these conditions, MHC class I transfer is <25% of the level when freeze-thawed cells and DC are mixed. To ensure that small molecules could traverse the transwell membrane, 50 nm FITC-coated latex beads were mixed with the freeze-thawed cells and added to one side of the transwells. After

three hours of culture, BMDC were analyzed for bead uptake. Only a partial reduction of bead uptake was observed (~29%, data not shown), indicating that small materials readily traversed the membrane. Collectively, these data suggest that optimal MHC class I transfer to DC requires direct contact with dead cells or large cellular debris, and is not efficiently mediated by soluble molecules or small vesicles such as exosomes (30-32) bearing MHC class I-peptide complexes.

MHC class I transfers from a variety of cells

To determine if DC acquisition of MHC class I was a general phenomenon, transfer experiments were conducted with the B16.BL6 8.2 melanoma (*H-2K^b*) and the A20 B lymphoma (*H-2^d*) cell lines. As seen in figure 2A, H-2K^b molecules from the melanoma cell line, and H-2D^d molecules from the B cell lymphoma were detected on BMDC from MHC mismatched (FVB) mice. Modest transfer to FVB BMDC was even seen from primary fibroblasts derived from the tail tissue of BALB/c (*H-2^d*) mice (figure 2A, right panel). These data in combination with previously published data (20) demonstrate that a wide variety of cell types can donate MHC class I molecules to DC.

To determine if the levels of MHC class I on the donor cell affected the efficiency of transfer, EL4/ova cells (*H-2^b*), which express very low levels of H-2K^b, were treated with IFNγ to up-regulate MHC class I expression (figure 2B). Freeze-thawed IFNγ-treated or untreated EL4/ova cells were then cultured with FVB-derived BMDC. Transfer of H-2K^b molecules from freeze-thawed IFNγ–treated EL4/ova cells was greater than from freeze-thawed untreated cells (figure 2C). Similarly, H-2D^d transfer from IFNγ–treated A20 cells to FVB BMDC was increased compared to non-IFNγ-treated A20

cells (data not shown). Increased transfer of H-2K^b from IFNγ–treated EL4/ova cells to splenic DC was also observed (data not shown). Therefore, increased expression of MHC class I on donor cells leads to enhanced acquisition of MHC class I proteins by DC.

Transferred peptide-MHC class I complexes activate CD8$^+$ T cells

The MHC class I molecules acquired by DC may activate T cells. To test this hypothesis, FVB BMDC were cultured with freeze-thawed EL4 cells transfected with the OVA gene (EL4/ova), purified, and tested as antigen presenting cells (APC) with OVA-specific, H-2K^b-restricted, OT-I transgenic CD8$^+$ T cells. BMDC exposed to EL4/ova, but not EL4 or OVA protein, activated OT-I T cells as measured by IL-2 release (Figure 3A). Similar results were obtained using splenic DC (data not shown). Therefore, DC acquire MHC class I molecules and antigen from donor cells, and use the acquired material to prime CD8$^+$ T cells.

T cell activation in Figure 3A could be due to the transfer of MHC class I – OVA complexes formed in the donor EL4/ova cells, or to the independent transfer of MHC class I and antigen and the subsequent assembly of MHC class I-peptide complexes in the recipient DC. To distinguish these two possibilities, FVB BMDC were cultured for three hours with either freeze-thawed EL4/ova cells or a combination of freeze-thawed EL4 cells and OVA protein. BMDC were purified and then tested for their ability to activate OT-I T cells. OT-I T cell activation only occurred if EL4 cells contained OVA (figure 3B), indicating that peptide-MHC I complexes are generated in the donor cells prior to transfer, and that the transferred complexes are presented by DC without further processing.

Cross-dressed DC efficiently activate CD8$^+$ T cells

We reported similar transfer of peptide-MHC II complexes in a previous study, and referred to the process as "DC cross-dressing" (20), a term originally coined by Yewdell and colleagues (33). To determine the role of cross-dressing in T cell priming, T cells were activated under conditions in which the relative contributions of cross-priming and cross-dressing could be assessed. C57BL/6 (*H-2^b*) BMDC were cultured with freeze-thawed DAP/ova or DAP/ova/K^b cells, purified by passage over ficoll, mixed with OT-I T cells, and T cell activation measured by assessing expression of the early activation marker CD69 (34) and by production of IL-2 and IFNγ. BMDC exposed to freeze-thawed DAP/ova/K^b cells induced a four-fold increase in the percent of CD69$^+$ OT-I T cells relative to BMDC exposed to freeze-thawed DAP/ova cells (figure 4A), while OT-I T cells cultured alone were less than 1% CD69$^+$ (figure 4A). IL-2 and IFN-γ production was similarly higher in cultures with DC exposed to freeze-thawed DAP/ova/K^b cells (figures 4B and 4C, respectively). No IL-2 release was observed when OT-I T cells were cultured with freeze-thawed DAP/ova/K^b cells in the absence of DC (figure 4B), indicating that DC are essential for CD8$^+$ T cell activation and that OT-I activation is not due to direct priming by the freeze-thawed DAP/ova/K^b cells. Comparable results were obtained if splenic DC were used as recipients, although fewer T cells expressed CD69 and both IL-2 and IFN-γ release was lower (data not shown). To determine if the differences in T cell activation were due to quantitative differences in cell-associated OVA, cell lysates of DAP/ova/K^b and DAP/ova cells were prepared and western blotted with antibodies to OVA. As shown in Figure 4D, DAP/ova cells contain

more OVA than DAP/ova/K^b cells, indicating that the enhanced activation of OT-I T cells by DC co-cultured with freeze-thawed DAP/ova/K^b cells is not due to differences in quantity of OVA protein.

The absence of significant T cell activation by DAP/ova-loaded DC vs. the strong T cell activation by DAP/ova/K^b-loaded DC (figure 4) is consistent with the hypothesis that cross-dressing is more efficient than cross-priming for low levels of soluble antigen. To explore this possibility, C57BL/6 BMDC were pulsed for three hours with soluble OVA at concentrations comparable to OVA levels in EL4/ova lysates (0.1 μg/ml), or with 100-fold excess OVA (10 μg/ml). The protein-pulsed DC were then compared to EL4 and EL4/ova-loaded DC for their ability to activate OT-I T cells. EL4/ova-loaded DC and DC pulsed with 100-fold excess OVA significantly activate OT-I T cells; however, DC pulsed with 0.1 μg/ml OVA do not activate (Figure 5). Therefore, cross-dressing is more efficient than cross-priming when antigen levels are low.

DC cross-dressing primes $CD8^+$ T cells in vivo

To determine if $CD8^+$ T cells are primed in vivo by cross-dressed DC, C57BL/6 mice were adoptively transferred with CFSE-labeled OT-I $CD8^+$ T cells and inoculated s.c in the lower lateral abdominal area with a 1:1 mixture of live and freeze-thawed DAP/ova/K^b cells or a 1:1 mixture of live and freeze-thawed DAP/ova cells. Three days later the draining lymph node was removed and examined for CFSE dilution. Inoculation of DAP/ova/K^b cells resulted in strong T cell priming (figure 6A, left panel). If cross-priming was occurring, then mice inoculated with H-$2K^b$-negative DAP/ova cells should display significant OT-I division; however, the response to DAP/ova is minimal (figure

6A, middle panel). OT-I expansion in response to DAP/ova/K^b cells could be due to cross-dressing or to direct presentation of OVA by DAP/ova/K^b cells. If DAP/ova/K^b fibroblasts are directly presenting OVA, then host DC would not be required for OT-I activation. To determine if DC are essential, CD11c-DTR mice (*H-2*^{b}), which, when treated with diphtheria toxin (DTx), are transiently depleted for CD11c expressing DC (35), were used. CD11c-DTR mice were DTx-treated, adoptively transferred with CFSE-labeled OT-I T cells, inoculated with a mixture of live and freeze-thawed DAP/ova/K^b cells, and three days later the draining lymph node examined for expansion of OT-I T cells. Deletion of $CD11c^+$ cells virtually eliminated expansion of OT-I T cells (figure 6A, right panel), demonstrating that DC are essential for T cell priming and that DAP/ova/K^b fibroblasts cannot directly prime OT-1 T cells. In contrast, OT-I expansion in non-diphtheria-treated CD11c-DTR transgenic mice inoculated with DAP/ova/K^b cells, and in non-transgenic mice inoculated with DAP/ova/K^b and treated with DTx was similar to expansion in figure 6A, left panel (data not shown). Therefore, $CD11c^+$ cells are required for in vivo T cell priming, demonstrating that DAP/ova/K^b cells do not directly present antigen, and supporting the conclusion that cross-dressing occurs in vivo and enhances T cell priming compared to cross-priming alone.

Although OT-I T cells expand in mice inoculated with a mixture of live and freeze-thawed ("dead") DAP/ova/K^b cells, inoculation with exclusively live or freeze-thawed DAP/ova/K cells did not induce significant OT-I T cell division (figure 6B), suggesting synergy between the live and freeze-thawed cell populations. To determine which cell population was providing the peptide-MHC complexes for cross-dressing, C57BL/6 mice were adoptively transferred with OT-I T cells and inoculated with 1:1

mixtures of live and freeze-thawed (dead) DAP/ova, DAP/ova/K^b, and NIH3T3 cells. OT-I T cells expanded in mice inoculated with a mixture of live DAP/ova and dead DAP/ova/K^b cells (figure 6C, left panel, but not in mice inoculated with mixtures of live DAP/ova/K^b plus dead DAP/ova cells, or mixtures of live NIH3T3 plus dead DAP/ova/K^b cells (figure 6C, middle and right panels). Therefore, T cell expansion requires live and dead cells, and the dead cells must contain the relevant peptide- MHC class I complexes while the live cells must contain the relevant antigen. As DC acquire peptide-MHC class I complexes from dead and not live cells (figures 1 and 3), the dead cell population is probably inducing DC cross-dressing, while the live population is inducing cross-priming. Therefore, maximum priming of $CD8^+$ T cells is probably the result of a combination of DC cross-dressing and cross-priming.

Discussion

The priming of $CD8^+$ T cells by cross-dressed DC is a newly identified mechanism by which $CD8^+$ T cells are activated. Although there has been speculation that cross-dressing occurs (33), this is the first report demonstrating that it takes place in vitro and in vivo. Cross-priming and cross-presentation, the generally accepted mechanisms for activating $CD8^+$ T cells, differ significantly from cross-dressing. Cross-dressed DC acquire peptide-MHC class I complexes from exogenous sources and present the complexes without further processing. Although DC also acquire antigen from exogenous sources during cross-priming/presentation, they process the acquired antigen intracellularly and present it in the context of their MHC class I molecules. Therefore, cross-priming activates $CD8^+$ T cells that are restricted to the MHC genotype of the DC and specific for peptides generated by the DC, while cross-dressing activates $CD8^+$ T cells that are restricted to the MHC genotype of the donor cells and are specific for peptides generated by the donor cells.

Several reports suggest that cells other than DC, such as tumor cells or fibroblasts, can directly activate $CD8^+$ T cells (6, 18, 19). These studies used mice inoculated with genetically mis-matched cells or recipient mice deficient in TAP so as to prevent cross-priming. However, DC were present in recipient mice and therefore could have been cross-dressed with peptide-MHC class I complexes from the inoculated cells. Therefore, to definitively demonstrate direct antigen presentation to $CD8^+$ T cells by non-professional APC, these studies should be done under conditions that prevent DC cross-dressing.

Although cross-priming and cross-presentation have been experimentally documented for many antigens, including soluble proteins and viral and tumor antigens, (reviewed in (2, 5)), not all $CD8^+$ T cell determinants are efficiently cross-presented (13, 18, 36, 37), suggesting that additional activation mechanisms are operating. Although direct presentation has been proposed as the relevant mechanism for epitopes that are inefficiently cross-presented (14), cross-dressing may be responsible. This conclusion is supported by the studies reported here demonstrating that cross-dressed DC prime $CD8^+$ T cells when cross-priming is inefficient. Although the relative contributions of cross-dressing and cross-priming to overall $CD8^+$ T cell activation may vary for different antigens, it is likely that a combination of the two mechanisms facilitates maximum $CD8^+$ T cell activation.

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Figure 1. MHC class I molecules transfer from donor cells to DC. *A.* FS x SS log profile, and PI and CD11c staining of bitmapped FVB (*H-2*^{q}) BMDC cultured for three hours with freeze-thawed DAP/ova cells, purified by passage over a ficoll gradient (top row). FS x SS log and PI staining of freeze-thawed DAP/ova cells (bottom row). *B.* FVB BMDC were cultured alone or with freeze-thawed DAP/ova cells, and analyzed for H-2K^{k} (top left histogram; isotype control staining top right histogram). BMDC were incubated alone or with live DAP/ova cells, and analyzed for H-2K^{k} (bottom left histogram). FVB BMDC were cultured alone or with freeze-thawed DAP/ova cells, purified, cultured overnight with LPS and anti CD40 mAbs, and analyzed for H-2K^{k} expression the following day. *C.* DAP/ova or H-2K^{b}-transfected DAP/ova (DAP/ova/K^{b}) cells stained for H-2K^{b}. *D.* FVB BMDC were cultured alone or with freeze-thawed DAP/ova (left histogram), or with DAP/ova/K^{b} (right histogram) cells, purified and analyzed for H-2K^{b}. *E.* FVB BMDC were cultured alone, with freeze-thawed DAP/ova cells, with the supernatant of freeze-thawed DAP/ova cells, or with a transwell separating DC from freeze-thawed DAP/ova cells. DC were subsequently analyzed for H-2K^{k}. All BMDC were purified by passage over ficoll gradients prior to antibody staining. BMDC histograms show the antibody staining for CD11c^{+} cells. These data are representative of three independent experiments.

Figure 2. MHC class I molecules transfer to DC from a variety of cells. *A.* FVB BMDC were incubated alone or with freeze-thawed B16, A20, or BALB/c fibroblast cells, and analyzed for donor MHC class I. *B.* EL4/ova or IFN-γ-treated EL4-ova cells stained for H-2K^{b}. *C.* FVB BMDC were incubated alone or with freeze-thawed EL4/ova or IFN-γ-

treated EL4-ova cells and analyzed for H-2K^b. These data are representative of three independent experiments.

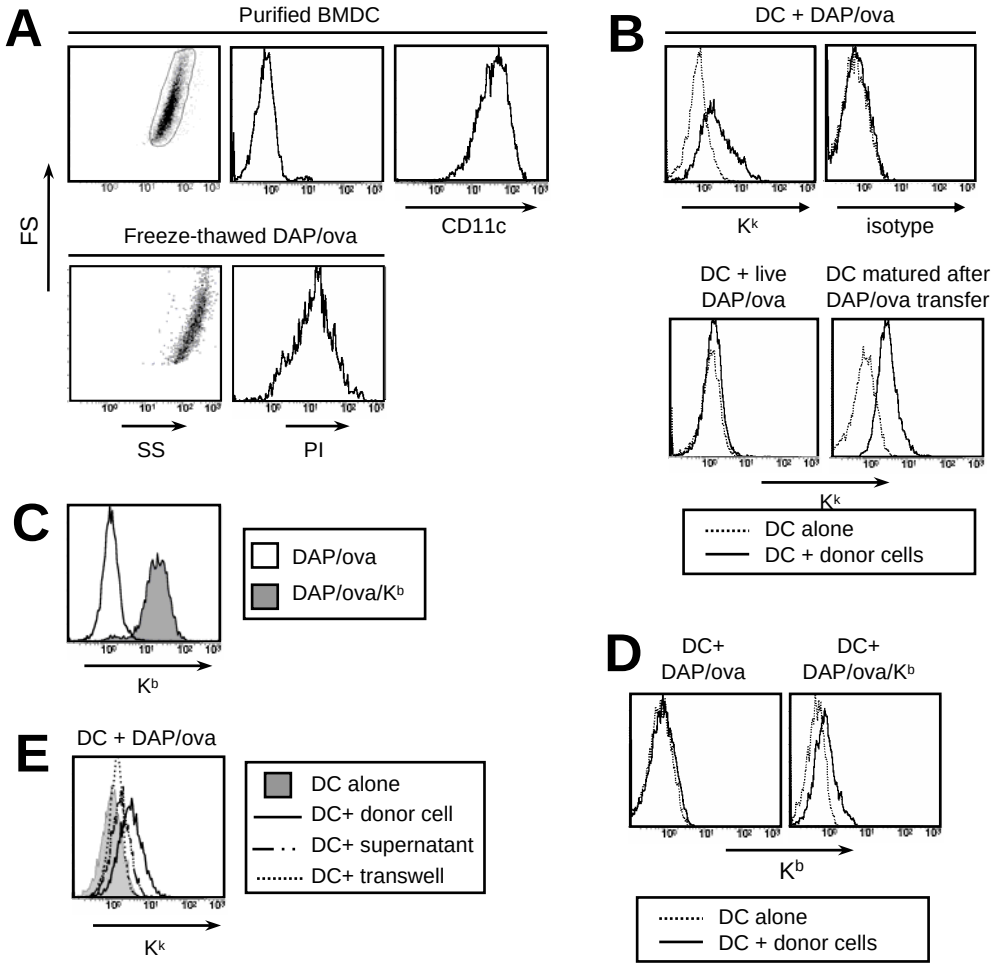
Figure 3. Peptide-MHC class I complexes transfer from donor cells to DC and activate CD8$^+$ T cells. *A.* FVB BMDC were cultured alone, with freeze-thawed EL4 or EL4/ova cells, or with 10 μg/ml OVA, purified by passage over a ficoll gradient, and cultured overnight with OT-I T cells. IL-2 release was quantified by ELISA. *B.* FVB BMDC were cultured with freeze-thawed EL4/ova cells or with freeze-thawed EL4 cells and 10 μg/ml OVA, purified, and cultured overnight with OT-I T cells. IL-2 release was quantified by ELISA. These data are representative of three independent experiments.

Figure 4. Cross-dressed DC activate naïve CD8$^+$ T cells. C57BL/6 BMDC cultured with freeze-thawed DAP/ova cells, DAP/ova/K^b cells, or without fibroblasts were purified on ficoll gradients and cultured with OT-I T cells. OT-I T cell activation was measured by CD69 expression at the indicated times *(A)*, or by IL-2 *(B)* or IFN-γ *(C)* production the following day. (*D*) DAP/ova and DAP/ova/K^b cells have similar levels of cell-associated OVA as determined by western blot. These data are representative of three independent experiments.

Figure 5. Cross-dressing efficiently activates CD8$^+$ T cells to low levels of soluble antigen. C57BL/6 BMDC were cultured with 0.1 or 10 μg/ml OVA, or with freeze-thawed EL4 or EL4/ova cells, purified on ficoll gradients, cultured overnight with OT-I

T cells, and T cell activation assessed by CD69 expression (*A*), or by IL-2 (*B*)or IFN-γ (*C*) release. These data are representative of three independent experiments.

Figure 6. Cross-dressed DC activate $CD8^+$ T cells in vivo. Recipient mice were adoptively transferred with CFSE-labeled OT-I T cells and 8-24 hours later inoculated s.c. with DAP transfectants. Three days later the draining lymph node was removed and examined for dilution of CFSE. Each histogram shows the result of an individual mouse. The average proliferation index ± S.D. for three mice tested in independent experiments is shown in the top left corner of each histogram. Proliferation indices marked with an * are significantly different from the unmarked indices ($p < 0.05$) and are not significantly different from each other. *A.* CFSE staining for C57BL/6 mice inoculated with a 1:1 mixture of live and freeze-thawed (dead) DAP/ova/K^b or DAP/ova cells (left and middle panels) or CD11c-DTR (*H-2*^{b}) mice depleted for DC by DTx injection and inoculated with a 1:1 mixture of live and dead DAP/ova/K^b cells (right panel). *B.* CFSE staining for C57BL/6 mice inoculated with live or dead DAP/ova/K^b cells, or a 1:1 mixture of live and dead DAP/ova/K^b cells. *C.* CFSE staining for C57BL/6 mice inoculated with a 1:1 mixture of live and dead DAP/ova, DAP/ova/K^b, or NIH3T3 cells.


A
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DC + DAP/ova
Kᵏ
isotype
DC + live DAP/ova
DC matured after DAP/ova transfer
Kᵏ
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DC + donor cells
C
DAP/ova
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DC + donor cells
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DC + DAP/ova
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DC alone
DC+ donor cell
DC+ supernatant
DC+ transwell

Figure 1

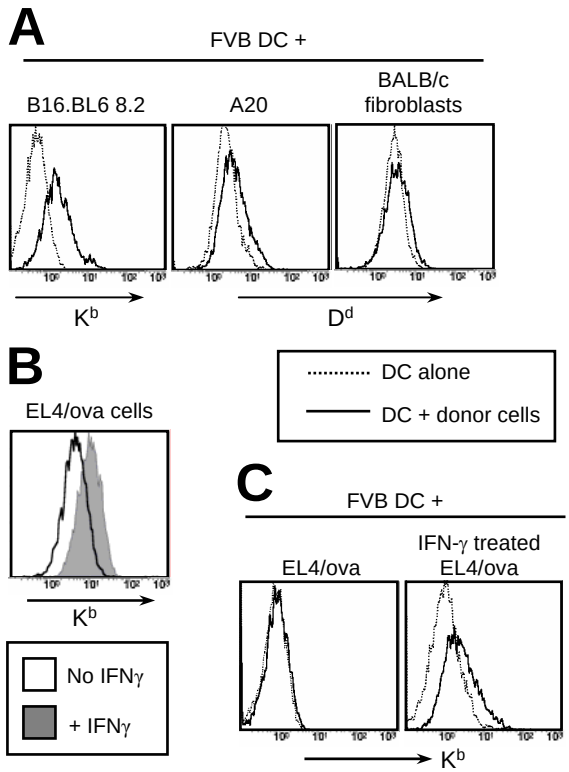
A
FVB DC +
B16.BL6 8.2
A20
BALB/c fibroblasts
Kb
Dd
B
DC alone
DC + donor cells
EL4/ova cells
Kb
No IFNγ
+ IFNγ
C
FVB DC +
EL4/ova
IFN-γ treated EL4/ova
Kb

Figure 2

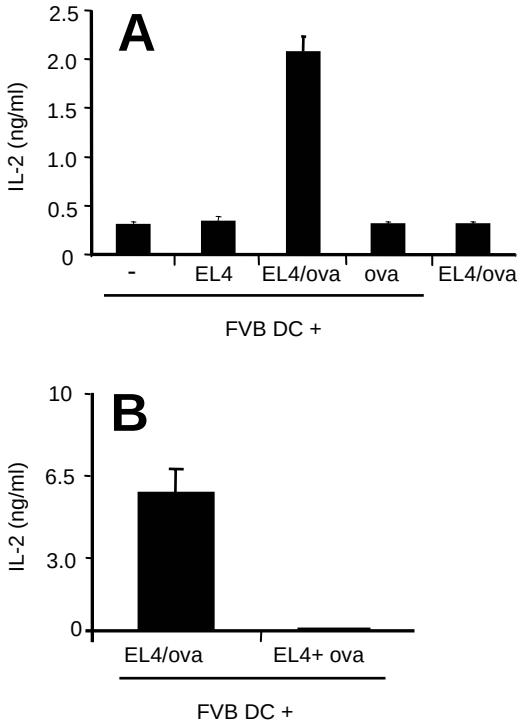
A
IL-2 (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
-
EL4
EL4/ova
ova
EL4/ova
FVB DC +
B
IL-2 (ng/ml)
10
6.5
3.0
0
EL4/ova
EL4+ ova
FVB DC +

Figure 3

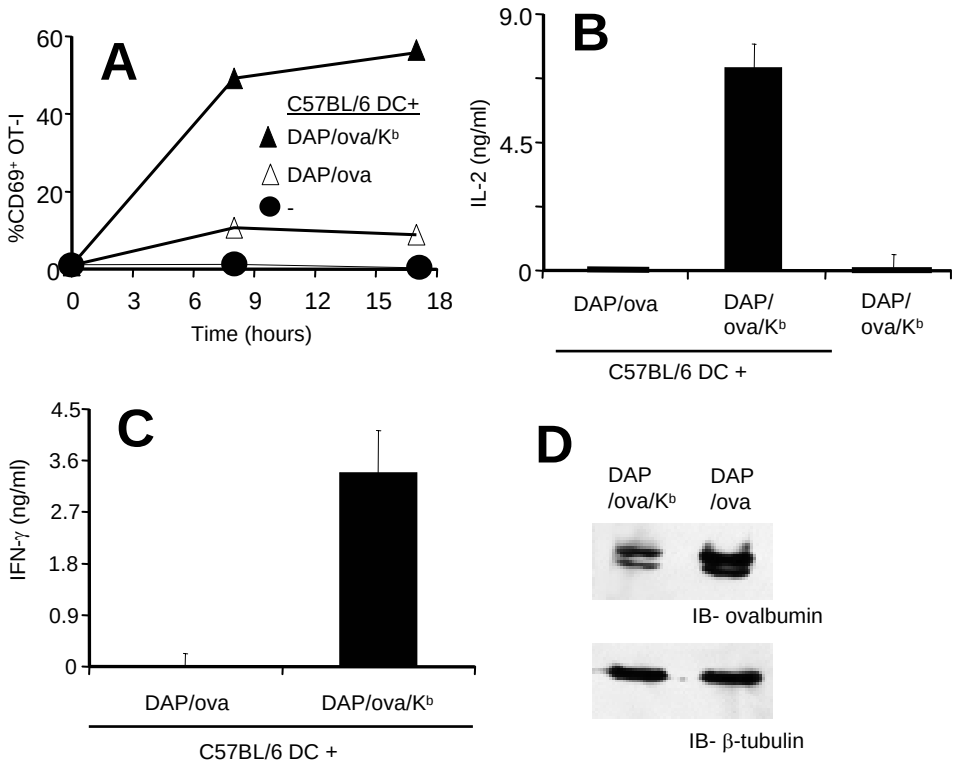
A
%CD69+ OT-I
60
40
20
0
0 3 6 9 12 15 18
Time (hours)
C57BL/6 DC+
DAP/ova/Kb
DAP/ova
-
B
IL-2 (ng/ml)
9.0
4.5
0
DAP/ova
DAP/ ova/Kb
DAP/ ova/Kb
C57BL/6 DC +
C
IFN-γ (ng/ml)
4.5
3.6
2.7
1.8
0.9
0
DAP/ova
DAP/ova/Kb
C57BL/6 DC +
D
DAP /ova/Kb
DAP /ova
IB- ovalbumin
IB- β-tubulin

Figure 4

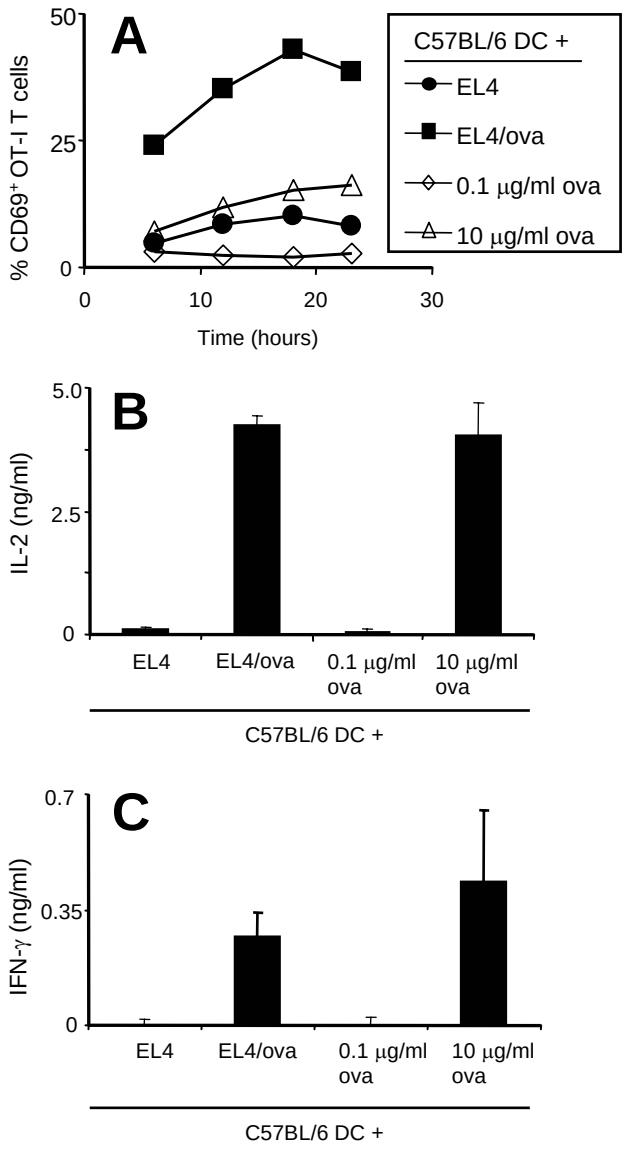
A
50
25
0
% CD69+ OT-I T cells
0
10
20
30
Time (hours)
C57BL/6 DC +
EL4
EL4/ova
0.1 μg/ml ova
10 μg/ml ova
B
5.0
2.5
0
IL-2 (ng/ml)
EL4
EL4/ova
0.1 μg/ml ova
10 μg/ml ova
C57BL/6 DC +
C
0.7
0.35
0
IFN-γ (ng/ml)
EL4
EL4/ova
0.1 μg/ml ova
10 μg/ml ova
C57BL/6 DC +

Figure 5

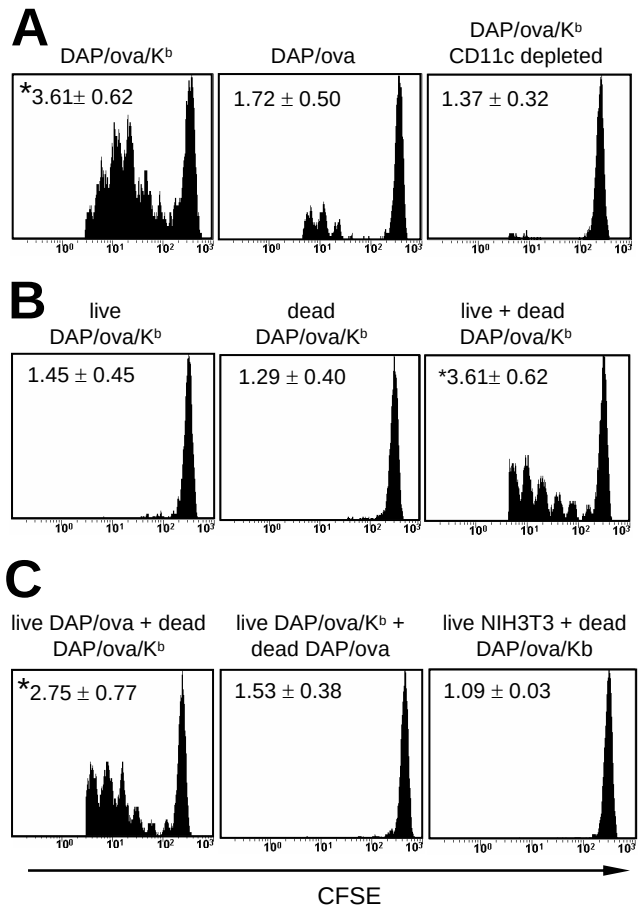
A
DAP/ova/Kb
DAP/ova
DAP/ova/Kb
CD11c depleted
*3.61± 0.62
1.72 ± 0.50
1.37 ± 0.32
B
live
DAP/ova/Kb
dead
DAP/ova/Kb
live + dead
DAP/ova/Kb
1.45 ± 0.45
1.29 ± 0.40
*3.61± 0.62
C
live DAP/ova + dead
DAP/ova/Kb
live DAP/ova/Kb +
dead DAP/ova
live NIH3T3 + dead
DAP/ova/Kb
*2.75 ± 0.77
1.53 ± 0.38
1.09 ± 0.03
CFSE

Figure 6